THE GROWTH IN TISSUE CULTURE OF TWO MEMBERS OF THE MYXOVIRUS GROUP

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by

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"All eternity might not be long
enough for such an extraordinary
system to arise by chance in
an inanimate world."

Hoyle, 1953.

CELL-VIRUS RELATIONSHIPS

From the considerable amount of experimental evidence which has accumulated over recent years, it is now possible to obtain a fairly accurate picture of the structure and general pattern of multiplication of many viruses; the reproductive cycles of the various groups have been shown to differ widely, but it is well-accepted that each cycle is dependent on living cells for the production of new infectious virus. A very close relationship thus exists between host cell and virus, and before attempting to explain fully the basic process of virus multiplication, all the mechanisms occurring during virus infection of a cell must be understood.

The most complete analysis of cell-virus relationships has been carried out with the bacterium-bacteriophage system which provides a relatively simple model allowing for accurate quantitative methods. The animal viruses represent a more complex situation; the development of tissue culture and chemically defined media have, however, made possible the study of the growth of these viruses and the reaction of the host cells during the infectious process.

A review is presented here of literature pertaining to the general field of host cell-animal virus relationships with particular reference to the Myxovirus group.

TISSUE CULTURE

The first recorded instance of the growth of living cells

in vitro, now known as tissue culture, was reported at the end of the last century, when Roux (1885) succeeded in maintaining the medullary plate of the chick embryo in warm saline. The true beginning of tissue culture was, however, marked by the experiment carried out by Harrison (1907), in which it was demonstrated that the normal functions of cells continue *in vitro*.

At this stage, the era of virology was also beginning; the actual discovery of viruses being attributed to Iwanowski (1892) although confirmation of his experiments was not obtained until Beijerinck (1899) proved without doubt that the agent of tobacco mosaic disease was filterable. At the same time, Loeffler and Frosch (1898) discovered that the agent of foot-and-mouth disease was also filterable, and soon after the turn of the century many viruses affecting plants and animals had been isolated.

But tissue culture developed more slowly due, partly, to many setbacks encountered with bacterial contamination. It was not until many years later that Carrel (1928) saw the possibility of using cultured cells in investigations of the relationship between virus and host cell. He demonstrated that animal cells could be grown indefinitely *in vitro* and from his work with Rous sarcoma virus he emphasised the following facts:

a) cells in a tissue culture infected with a virus may show alterations in their behaviour which may be directly observed;

b) large quantities of virus may be generated in the culture and this generation may continue indefinitely,

c) certain viruses may establish within the cells a relationship that may be regarded as essentially symbiotic;

d) one type of cell may be susceptible to virus infection and another resistant.

The importance of these observations is now evident but before they were applied to any extent, techniques had to be improved and simplified. Many different tissues were grown in vitro and the development of continuous cell lines was of particular practical importance, especially the isolation of abnormal strains, e.g. strain HeLa (Gey, Goffmann and Kubicek, 1952), which could be grown easily in culture and supported the multiplication of many viruses. These developments have been of great value, particularly in routine virus diagnosis, and the use of animals has consequently decreased. Many methods of growing cells in vitro have evolved; the development of the roller tube (Gey, 1933) was of considerable importance in virology, as neutralisation tests and virus and biochemical assays could easily be carried out. Recently, an excellent quantitative method has been provided by the plaque technique (Dulbecco and Vogt, 1954) whereby virus can be measured accurately by the number of plaques produced in a cell monolayer.

If infected cells are to be studied microscopically, a

culture chamber embodying good optical properties is essential, and it is also of considerable advantage if some means of perfusion is incorporated. Many such chambers have been devised (Carrel, 1931; de Haan, 1931; 1938; Lindbergh, 1939; Parker, 1950) and a few have been designed specifically for use in microcinematography in conjunction with phase-contrast or interference microscopy (Hu et al., 1951; Pomerat, 1951; Christiansen et al., 1953; Schwöbel, 1954, 1955; Dick, 1955; Richter and Woodward, 1955).

With the development of new techniques, the use of chemically defined media, and the possibility of incorporating antibiotics into the medium, it is now possible, not only to investigate the multiplication of a virus within a particular cell system, but also to attempt to correlate this multiplication with changes within the cells, both morphological and cytochemical.

VIRUSES IN TISSUE CULTURE

Morphological changes in virus-infected cells were reported in the early days of tissue culture (Andrewes, 1929; Rivers et al., 1929; Huang, 1943) and although such changes were employed in neutralisation tests, the significance of these changes was not widely recognised. However, the demonstration by Enders and his colleagues (Enders et al.,1949; Weller et al., 1949; Robbins et al., 1950) that the changes induced in cultures of extraneural tissues by polio virus were actually due to the presence of the virus has not only proved

the importance of tissue culture in the diagnosis of virus disease but has furthermore stimulated renewed interest in the field of cell-virus relationships.

Cytopathogenic effect.

The ability of a virus to induce changes in tissue culture is known as its "cytopathogenicity" and this term has been broadly defined by Enders (1954) as "the capacity to induce any demonstrable departure from the normal either in the morphological or functional properties of the cell". The terms "cytopathogenic" and "cytopathic" are, however, often used indiscriminately and it has recently been suggested by Tyrrell et al. (1959) that "cytopathic" should be used in preference to "cytopathogenic" to mean "causing or associated with cell destruction".

Morphological changes

Classification of changes. In his review, Enders (1954) classifies the morphological changes observed in cells into three groups:

Group 1:- rounding or assumption of irregular or bizarre outlines, granulation of the cytoplasm, swelling of the cell, nuclear swelling, pyknosis and fragmentation of the nuclear chromatin, cell death and disintegration.

Group 2:- formation of inclusion bodies in either cytoplasm or nucleus.

Group 3:- formation of giant cells or coalescence of cells into syncytial masses.

In this way viral agents can be classified according to their cytopathic effect. For example, the adenoviruses (Hilleman and Werner, 1954), the Coxsackie viruses (Riordan et al., 1952) and the polio viruses (Robbins et al., 1950) can be classified in group 1; vaccinia (Rivers et al., 1929), virus 111 (Andrewes, 1929) and psittacosis (Bedson and Bland, 1932) in group 2; measles virus (Enders and Peebles, 1954), herpes simplex (Rivers et al., 1929) and certain simian viruses (Ruckle, 1958) in group 3. All three types of change may even be produced in the same virus-cell system.

Functional disturbances

As mentioned previously, the "cytopathogenic effect" of a virus refers not only to the morphological changes induced in the cells but also to the functional disturbances. Early reports of such changes have been few, but during the last decade, many workers have investigated the metabolic alterations occurring in virus-infected cells.

Since the demonstration by Gierer and Schramm (1956) that ribonucleic acid (RNA) obtained from tobacco mosaic virus was infective, many examples of the isolation of infectious RNA from animal viruses have been reported. These include polio and West Nile encephalitis (Colter et al., 1957 a), Mengo encephalitis (Colter et al., 1957 b), equine encephalomyelitis (Wecker and Schäfer, 1957), murine encephalo-

myocarditis (Huppert and Sanders, 1958 a, b), foot-and-mouth disease (Brown et al., 1958), murine encephalomyelitis (Franklin et al., 1959), influenza (Maassab, 1959) and certain enteroviruses (Sprunt et al., 1959). These findings have given rise to much speculation over the origin of the infectious RNA isolated; it may possibly arise from non-infective virus materials (Brown et al., 1958; Huppert and Sanders, 1958 b). In experiments with influenza virus containing radioactive nucleic acid, Hoyle and Frisch-Niggemeyer (1955) demonstrated that most of the nucleic acid became bound to insoluble cell components and part of the remainder was liberated as ribonucleoprotein complement-fixing antigen.

Considering that new materials are being formed in virus-infected cells, it is not altogether surprising to find many metabolic changes taking place within these cells. Particular attention has been directed towards phosphorus uptake and other changes related to nucleic acid distribution. Womack and Kass (1953) observed rapid incorporation of radio-active phosphorus by influenza-infected chick embryo cells, and Kovacs (1956) noted the alteration of phosphatase activity in monkey kidney cells infected with P32 labelled polio virus. In HeLa cells, large increases have been found in the cytoplasmic RNA after polio virus infection (Maassab et al., 1957) and in the nuclear deoxyribonucleic acid (DNA) after infection with herpes virus (Newton and Stoker, 1958). These increases were found to be greater than could be accounted for by the nucleic acid in the newly-formed virus and furthermore, the RNA in the polio-

infected cells had the composition characteristic of the host rather than that of the virus (Ackermann, 1958).

It has been suggested in a more recent paper (Ackermann et al., 1959) that different viruses condition different metabolic areas to synthetic activity; for example, polio virus induces RNA production in the cytoplasm, whereas herpes induces the production of DNA in the nucleus. These metabolic changes could account for abnormal growth or death of the infected cells.

An important advance, which is helping to relate morphological changes in virus-infected cells with cytochemical changes, has been the development of the acridine orange staining technique (Strugger, 1940) for use in virology (Armstrong and Niven, 1957). After treating cells with acridine orange, it is possible, by means of fluorescent microscopy, to identify the distribution of nucleic acids within the cells, and further, to distinguish between RNA and DNA; nuclear chromatin containing DNA emits greenish-yellow fluorescence, whereas the parts of the cytoplasm and the nucleolus which have an appreciable content of RNA fluoresce flame red. Many virus - cell systems have been studied with this technique and it has been found that the changes in nucleic acid pattern following virus infection are of four kinds (Niven, 1959). It seems significant that in all cases where the nucleic acid constituent of a virus agent is known, the

dominant change has occurred in that nucleic acid type; for example, with the RNA-containing influenza virus the dominant change detected by the acridine orange method was the development of RNA in the nucleus. At the moment, however, there is no justification for assuming that this unusually situated nucleic acid is virus nucleic acid unless the evidence is supported by immunological data such as is obtained by the Coons fluorescent antibody technique.

The fluorescent antibody technique, in which an antigen can be "stained" by means of antibody coupled to a fluorescent dye (Coons and Kaplan, 1950), provides an effective method of studying the intracellular location and development of virus antigens. Several viruses have been investigated in this way, for example, mumps (Watson, 1952), influenza (Watson and Coons, 1954; Liu, 1955) and vaccinia (Noyes and Watson, 1955); the technique is proving particularly useful in investigations on the nature of virus multiplication, as in the case of fowl plague virus (Breitenfeld and Schäfer, 1957). In many of these experiments, fluorescein isocyanate has been used to label the antisera but the use of compounds such as Lissamine Rhodamine B200 (Chadwick et al., 1958), which possesses a fluorescent colour contrasting strongly with the autofluorescence occurring in normal tissues, has obvious advantages.

Correlation between cytopathic effect and viral replication.

The cytopathic changes induced by viruses in tissue culture are now widely used in routine diagnostic work but the relationship between them and actual viral multiplication has not yet been completely elucidated. In many recent studies on virus cell interaction, the correlation between replication of virus and concurrent cellular changes has been emphasised. Studies on vaccinia in HeLa cells (Ryden and Randall, 1957) and on polio in monkey kidney cells (Reissig et al., 1956) and in HeLa cells (Harding et al., 1956) provide evidence that increase in extracellular virus is correlated with progressive cytopathic changes. This is quite probable but why does the production of virus result in cellular destruction?

Although Enders classifies the morphological disturbances produced by viruses into three groups he points out that these changes may be induced by many agents and cannot necessarily be considered as the direct result of viral activity. Many factors are responsible; the species from which the cells are derived is probably the most important factor and, with many viruses, cell type may be equally important. For example, it has been demonstrated that a tumour cell is susceptible to the Eastern equine encephalitis virus, whereas the normal derivative is resistant (Bang and Gey, 1952). Environmental conditions, such as composition of medium and growth temperature, may also tend to enhance or suppress cytopathogenicity.

In order to explain the degenerative changes induced by many viruses it has been suggested that, following infection, the metabolism of the host cell is upset due to the depletion of some key nutrient. Support for this suggestion has been provided by Eagle (1955) who observed a similarity in appearance between those cells suffering from deficiencies of single amino-acids and those infected with certain viruses. In a recent review, Ackermann (1958) mentions the effect of 5-fluorouracil on HeLa cells; the function of thymine in the synthesis of DNA is blocked, thereby preventing cellular division, but cell growth continues and a giant cell is produced. On the basis of these findings, Ackermann suggests that cytopathic changes depend, not on the synthesis of foreign material, but on an unbalanced growth of the cell which may be the result of the action of a particular substance. Further evidence for this hypothesis has been provided by Pereira (1958) who reported the presence of a protein factor responsible for the early cytopathic changes induced by adenoviruses in HeLa cells. This early effect was reversible and was accompanied by changes in cellular viability or multiplication rate.

This hypothesis seems to provide an adequate explanation of the case where cellular changes appear concomitantly with increase of infectious virus. Other situations may occur, however, in which (a) cytopathic changes appear without increase of infectious virus, (b) persistent or latent infections occur

without apparent cellular damage, or (c) virus production occurs without any evidence of morphological changes.

Cellular damage without increase of infectious virus.

The occurrence of cellular injury in the absence of multiplication of infectious virus is usually attributed to the presence of a toxin. The toxicity of influenza virus has been thoroughly investigated (Henle and Henle, 1946) and there is evidence that the reaction is due in some cases to an abortive infection of the cells in which virus fails to reproduce to maturity, for example in the cells of the mouse brain (Schlesinger, 1950) and in HeLa cells (Henle et al., 1955). Such so-called incomplete cycles of multiplication will be discussed in more detail in a later section. A toxic effect has also been demonstrated in mouse lungs after inoculation with massive doses of Newcastle disease virus (Davenport, 1952); there was no evidence of any viral multiplication in this case.

Cellular injury without increase of infectious virus has been demonstrated experimentally in HeLa cells with type 3 polio virus (Ackermann et al., 1954). By means of the anti-metabolite fluorophenylalanine virus multiplication could be inhibited, but in spite of this the production of cytopathic changes continued. The recent report that an extraviral toxin is produced in polio-infected cells (Ackermann et al., 1958) may provide an explanation for the apparent autonomy existing between viral increase and cellular injury.

Persistent or latent infections.

In contrast to the situations mentioned above where

cellular injury occurs with or without virus increase there are many reports in the literature where virus could be isolated over long periods of time from apparently normal tissues.

Such examples of persistent or latent infections have been achieved by various means; few of these, however, represent a true symbiotic relationship between host cell and virus such as has been observed with Newcastle disease virus (NDV) in HeLa cells (Ciecura et al., 1957; Deinhardt and Henle, 1957). HeLa cells were exposed to NDV, and after single cell isolations had been carried out on the remaining cells, clonal stocks were grown and passed through hundreds of generations without exposure to the virus. After this, virus could still be demonstrated in the cultures, and when exposed to further infection, the cells were found to be more resistant than a culture of the original strain from which the cells were produced. The recent report of polio-resistant HeLa cells (Vogt and Dulbecco, 1958) may represent a similar situation.

It is difficult to separate a true symbiotic relationship from low-grade infections in which small numbers of cells are constantly being infected and destroyed. These may be described as chronic infections and have been observed in many virus-cell systems. For example, Chambers (1957) reported the continued production of Western equine encephalitis (WEE) virus by L cells over a period of many months, and during the time the cells were producing virus they were immune to mass

destruction by superinfection. Chronic infections have also been described for Eastern encephalomyelitis virus in various cultured cell strains (Bang et al., 1957), Coxsackie A9 in HeLa cells (Takemoto and Habel, 1959) and for foot-and-mouth disease virus in calf kidney cells (Dinter et al., 1959). Henle et al. (1958) have studied cultures of human cell lines (MCN and Lung-To) which were persistently infected with Newcastle disease virus and other viruses. The infected cells showed no spontaneous cell degeneration and they were resistant to the cytopathogenic action of viruses such as PR8, herpes simplex and vesicular stomatitis. In a more recent paper (Henle et al., 1959) these workers have suggested that this is an interference effect and report the production of a substance resembling interferon which may be responsible.

The many naturally-occurring latent infections have been reviewed by Stoker (1957); of these, herpes simplex infection of man provides a classic example. About 60 per cent. of people are infected and remain carriers throughout life; such carriers possess antibody in the blood, but this does not lead to the elimination of the virus, which remains latent until activated in some way. The infection has recently been investigated in vitro (Fernandez, 1960) and it was found that HeLa cells, which had survived infection with herpes simplex, continued to carry the virus for at least nine months, even in the presence of antiserum. The cells could be re-infected but 10 - 100 times as much virus had to be present to infect a

recovered cell as to infect a stock HeLa cell. A rather similar situation has been obtained experimentally with polio virus (Ackermann and Kurtz, 1955). These workers showed that, if a culture of HeLa cells was heavily infected with poliomyelitis virus, most of the cells died but a few surviving cells grew out and these could be maintained as persistently infected cells. The presence of sufficient antibody was necessary to preserve the relationship, as in the case of the herpes simplex infection; in the presence of normal serum the cultures showed spontaneous cytopathic changes. In this system it appeared that the immune serum inhibited the cytopathic effect of the virus without completely eliminating the infection and thereby favoured the survival of the virus rather than its elimination.

A type of latent infection can also be achieved by altering the composition of the medium. Following studies on the factors essential for multiplication of psittacosis virus (Hare and Morgan, 1954), Morgan (1956) found that chick embryo tissues lost their capacity to support the multiplication of the virus after being maintained in Hank's solution; active multiplication of the virus occurred, however, after enriching the medium with beef embryo extract; the virus could be kept in a state of latency for as long as 15 days. Similar results were obtained with psittacosis in L cells (Morgan and Bader, 1957) and these workers were able to define chemically the constituents necessary for activation or repression of infection. On the basis of their results they suggest that cell nutrition,

as well as an alteration in the immunological defences of the host, may prove an important factor in the activation of latent viral infections. Furthermore, the selection of partially resistant host cells and the ability to produce such a substance as interferon may also have to be considered.

Virus production without evidence of morphological changes.

At this stage a final situation must be mentioned in which virus production may occur normally without any evidence of morphological changes in the host cells - the Rous sarcoma virus (Rubin, 1956) provides a good example.

Eventually the accumulating knowledge concerning the relationship between virus and cell should have a practical application but it is essential at this point to consider the extent to which this relationship may be determined by the environment and that the results obtained *in vitro* may not always be comparable with those occurring *in vivo*. In this connection a striking example has been given by Kaplan (1955) with polio virus and monkey kidney cells. When monkey kidneys were inoculated directly with polio virus *in vivo*, no cytopathic changes could be found and infective virus could not be recovered from the kidneys or from cellular homogenates. However, after trypsinising such kidneys and plating out the cell suspensions as tissue cultures, cellular degeneration occurred with the release of new infective virus. Virus multiplication and cytopathic changes thus became evident only when the cells were removed to an alien environment.

THE MYXOVIRUS GROUP.

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General Properties.

The properties and nomenclature of the so-called Myxovirus group, which includes the influenza viruses (M. influenza A, B and C), fowl plague virus (M. pestis-galli), mumps virus (M. parotidis) and Newcastle disease virus or NDV (M. multiforme) have been described in some detail (Andrewes et al., 1955). The parainfluenza viruses (Andrewes et al., 1959) fall into the same general classification.

The members of this group resemble each other in size, susceptibility to ether, ability to multiply in the amniotic cavity of the chick embryo, ability to agglutinate mammalian or avian erythrocytes and presence of a viral receptor-destroying enzyme. It has recently been suggested by Andrewes (1959) that the parainfluenza viruses seem to form, with mumps and NDV, a subgroup of myxoviruses differing from the true influenza viruses in their rather larger size (Dawson and Elford, 1949) and a capacity to lyse as well as agglutinate erythrocytes (Burnet, 1949). The myxoviruses also possess a complement-fixing antigen. The members of the group can thus be distinguished by complement-fixation tests (Fulton and Dumbell, 1949) and haemagglutination-inhibition tests (Hirst, 1942 a).

Haemagglutination.

The discovery of the haemagglutinating property of the influenza viruses has been responsible, to a large extent, for

the vast amount of research which has been carried out in recent years on this group of viruses. The reaction was first recognised by Hirst (1941) simultaneously with McLelland and Hare (1941); these workers found that the allantoic fluid from chick embryos, previously infected with strains of influenza A virus, could agglutinate the red cells from the ruptured vessels in the egg. The importance of this finding was at once realised and a quantitative method for measuring influenza virus was rapidly worked out (Hirst, 1942 a; Salk, 1944); mouse protection tests were thus eliminated.

Although the myxoviruses are able to agglutinate red cells of a variety of different animals, for example, chicken, guinea-pig and man, there may exist variations among red blood cells of individual animals and even among strains of the same virus. This is exemplified by the O-D variation originally observed by Burnet and Bull (1943), in which it was found that newly isolated strains of influenza A virus showed variation in their haemagglutinating capacity after passage in the chick embryo; the original O form became a derived or D form which gave a higher ratio between the haemagglutinating titre for chicken red cells and that for guinea-pig red cells.

The haemagglutination reaction has been further developed for use in tissue culture in the form of the haemadsorption technique (Vogel and Shelokov, 1957). This was based on the finding that monkey kidney monolayers, when infected with an influenza virus, will adsorb guinea-pig or fowl erythrocytes. The test is read microscopically and has been of value in

detecting the presence of some members of the parainfluenza group of viruses. The reaction may be specifically inhibited by immune serum and may thus provide a rapid diagnostic and typing technique (Shelokov et al., 1958).

Haemagglutination is not confined to the myxovirus group and has been observed with the pox viruses (Chu, 1948), certain members of the ECHO and Coxsackie groups (Goldfield et al., 1957), and the arbor viruses (Casals and Brown, 1954). On the other hand, the myxoviruses may be distinguished from most other groups by the fact that they elute spontaneously after adsorption to red cells. This spontaneous elution was originally attributed by Hirst (1942 b) to the action of an enzyme system, and this was confirmed more recently when it was found that all members of this group possess an enzyme capable of splitting neuraminic acid derivatives from mucoproteins found on the surface of cells. There is still doubt, however, as to whether the action of this so-called mucinase is due to an enzyme (Bauer, 1953).

From experiments with influenza virus in ferret lungs in which adsorption of the virus similar to that obtained with red cells was observed, Hirst (1943) suggested that the enzymic activity of the virus on the host cell might represent a fundamental stage in infection. At the present time, the haemagglutinin of influenza virus is still thought to play an important part in the initiation of infection but the closely related fowl plague virus has been found to infect cells without

the action of its haemagglutinin (Schäfer, 1959).

Structure and Chemical Composition.

The structure and chemical composition of the myxoviruses are now understood in some detail; much of the research in this field has, however, been concentrated on influenza virus and the following description will be limited, for the most part, to this member of the group.

The influenza viruses normally occur as spherical particles of approximately 100 mμ diameter, A strains being somewhat smaller than B strains (Dawson and Elford, 1949), but in recently adapted strains filamentous forms may also be present (Chu, Dawson and Elford, 1949). Such forms have also been observed in tissue culture with the egg-adapted PR8 strain (Murphy et al., 1950). Although these filamentous forms are known to be associated with infection, they have not been shown to be infectious and there is still considerable controversy over their origin. The suggestion that they might represent an incomplete form of the virus was refuted by the finding that incomplete virus particles are spherical (Werner and Schlesinger, 1954), and it is now thought that the filament of influenza virus originates as an extrusion of a pathological cell (Bang and Isaacs, 1957).

Chemical analyses have shown the virus particle to contain approximately 60 per cent. protein (Knight, 1947), 30 per cent. lipid (Uhler and Gard, 1954) and 3.5 per cent. carbohydrate (Frisch-Niggemeyer and Hoyle, 1956; Frommhagen and Knight, 1956);

various estimates have, however, been put forward for the nucleic acid content of the virus. Early studies (Taylor, 1944; Knight, 1947) indicated the presence of both RNA and DNA, whereas more recently, Ada and Perry (1954) reported the presence of 0.86 per cent. RNA with no detectable DNA. In further studies, Frisch-Niggemeyer and Hoyle (1956) and Frommhagen and Knight (1956) obtained slightly less RNA (0.7 per cent.) than Ada and Perry, whereas Miller (1956) and Burke et al. (1957) found about 1 per cent. RNA and a small proportion of DNA.

On treatment with ether (Hoyle, 1952) the infectious particle disintegrates into two subunits, the complement-fixing soluble (S) antigen and the haemagglutinating viral (V) antigen. The S antigen of influenza virus was first described by Hoyle and Fairbrother in 1937 and received its name owing to the failure of these workers to sediment the antigen in the centrifuge. It appears to be analogous to the g or gebundenes antigen of fowl plague (Schäfer, 1957) and is immunologically group-specific. The antigen consists of spherical units about 10 - 12 mμ in diameter (Henle and Wiener, 1944; Hoyle et al., 1953) and chemical analyses have shown these units to be ribo-nucleoprotein containing 5.3 per cent. RNA (Hoyle et al., 1954; Hoyle and Frisch-Niggemeyer, 1955); the whole of the nucleic acid of the virus appears in this fraction, and considering the close link which exists between RNA and infectivity, the S antigen is probably responsible for the

infectivity of the elementary body. It has, in fact, been proved that incomplete virus contains less RNA and less S antigen than infectious particles (Ada and Perry, 1956; Lief and Henle, 1956 b).

The viral (V) antigen or haemagglutinin, which is strain specific, consists of units of about the same size as the S antigen (Hoyle et al., 1953) but these units are situated nearer the surface of the elementary body. Recently, Paucker et al. (1959) have described somewhat larger units. The units contain, besides protein, 4.2 per cent. carbohydrate, which may be a contaminant adhering to the surface of the particles. The V antigen or haemagglutinin should not be confused with the extracellular noninfectious haemagglutinins which will be discussed in the section on incomplete virus.

Influenza virus is also known to contain some host protein (Knight, 1946; Smith et al., 1955; Frisch-Niggemeyer, 1959) which forms part of its surface; this material is probably taken up by the virus during its liberation from the host cell. Recent experiments employing chromatography (Matheka and Armbruster, 1958) and electrophoresis (Henderson and Kempf, 1959) suggest that influenza virus may possess other antigenic substances.

An idea of the location, within the influenza virus particle, of the various components mentioned above may be obtained by examining Figs. 1 and 2. Although differing slightly in arrangement, the essential structure of these

hypothetical models is similar, i.e. a spherical particle with an inner core of ribonucleoprotein S antigen, and an outer layer of lipid near the surface of which occur protein haemagglutinin units. Frisch-Niggemeyer's model is slightly more detailed in that it shows the presence of protein "interfering substance" and also a possible position for host protein just within the carbohydrate outer membrane.

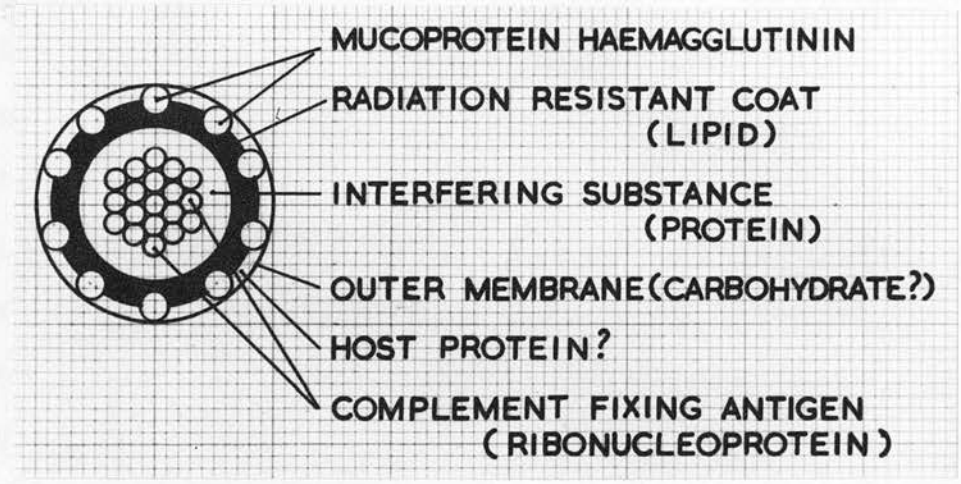

Figure 1.
The internal structure of influenza virus (Frisch-Niggemeyer, 1959).

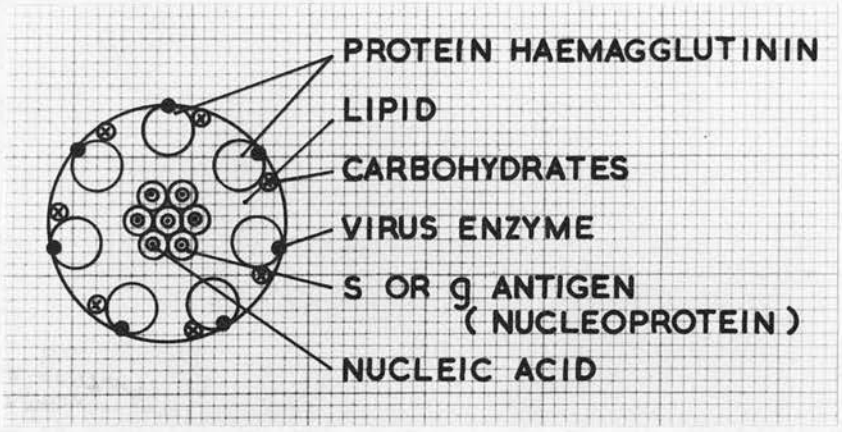

Figure 2.
The internal structure of influenza virus (Blascovic, 1959).

These diagrammatic representations of the structure of influenza virus are supported by chemical evidence and by evidence from electron microscope studies of the virus (Werner and Schlesinger, 1954; Birch-Andersen and Paucker, 1959) in which the particle was found to consist of an electron dense core, an inner membrane, an external coat of lower density and a less distinct external membrane. Incomplete virus, on the other hand, appeared flatter than standard virus, the electron dense centres were lacking and no internal details could be seen.

Other members of the myxovirus group may quite conceivably have a basic structure similar to that of influenza virus. They all occur normally as spherical particles, although filamentous forms may also be found, for example in the case of NDV (Bang, 1946); the virus of fowl plague has a diameter almost the same as that of influenza, whereas NDV and mumps are considerably larger (Dawson and Elford, 1949). The fowl plague virus may be degraded with ether by the method of Hoyle (1952) to give the g or gebundenes antigen and a haemagglutinin (Schäfer, 1957); these products are thought to correspond to the S and V antigens of influenza. Similar experiments have not been carried out with other members of the group; NDV is known, however, to contain a small noninfectious haemagglutinating component (Granoff et al., 1950) and resembles influenza in that it contains RNA but only a trace of DNA (Franklin et al., 1957).

BEHAVIOUR OF THE MYXOVIRUSES IN TISSUE CULTURE.

Influenza virus.

Culture.

Multiplication of influenza virus was first observed in ferrets (Smith et al., 1933), since when this animal has proved to be an excellent laboratory host. The virus was then adapted to mice (Andrewes et al., 1934) and later the chick embryo was found to be a most susceptible host (Burnet, 1940; Henle and Henle, 1944; Hirst, 1945; Beveridge and Burnet, 1946); de-embryonated eggs have also proved to be of value (Bernkopf, 1950).

Influenza virus was first grown in tissue culture twenty-five years ago when Francis and Magill (1935) grew the PR8 strain of influenza virus (Francis, 1934) in Li and Rivers' medium, i.e. minced chick embryo tissue in Tyrode's solution. The virus retained its infectivity for mice throughout twenty subcultures. But in spite of this success, reports of the *in vitro* growth of influenza virus have been few until the last few years. It had, in fact, been doubted whether influenza produced any cytopathic effect in tissue culture but Pollard and Bussel (1953), using a neurotropic strain of the virus, observed partial destruction of cells from human and rodent tumours. Soon after this, it was found that human kidney tissue culture could be used for the primary isolation of influenza virus (Mogabgab et al., 1954) and that monkey kidney was equally suitable (Mogabgab et al., 1955; Takemoto et al.,

1955); cytopathic changes were produced in these cells by the virus. The growth of the virus in monkey kidney cells has been further investigated (Mogabgab et al., 1956; Green et al., 1957 a, b) and kidney tissues from other species including calf kidney (Green et al., 1957 b; Haas and Wulff, 1957; Warren and Cutchins, 1957; Heath and Tyrrell, 1958), swine kidney (Wulff and Haas, 1957; Hinz and Syverton, 1959), chick embryo kidney (Heath and Tyrrell, 1958; Mannweiler, 1959), and rabbit kidney, guinea-pig kidney, hamster and mouse kidney (Heath and Tyrrell, 1958) have been used.

Although kidney tissues, especially those derived from monkeys and bovine embryos, have been most extensively used for the isolation and cultivation of influenza viruses, other tissues have also been employed. Cells from minced chick embryos (Daniels et al., 1952; Simpson and Mogabgab, 1957) from the allantoic membrane (Bate, 1955) and from chick embryo lung (Tyrrell, 1955; Ledinko et al., 1957) have been found to support growth of the virus, whereas no multiplication could be detected in chick embryo fibroblasts (Stulberg and Schapira, 1953). Embryo mouse lung (Gostling, 1958) and whole mouse embryos (Gostling, 1960) have also been shown to support the multiplication of infective influenza virus, but human conjunctival cells and KB carcinoma cells have not shown this capacity. The growth of influenza in the tissues mentioned above usually results in the production of infectious virus although the production of incomplete virus or non-infectious

haemagglutinins may be induced under certain conditions, for example in chick embryo lung cells (Ledinko et al., 1957) and in chick embryo kidney cells (Mannweiler, 1959).

A rather different situation occurs when HeLa cells are infected with influenza virus; although cytopathic changes occur with concomitant production of haemagglutinins, no rise in infective virus may be detected under any conditions (Henle et al., 1955; Tyrrell, 1955, Girardi et al., 1956; Green et al., 1957 b). The haemagglutinin is presumed to be newly produced due to the ready incorporation of radioactive phosphorus into these particles which suggests that an incomplete or abortive cycle of multiplication is taking place. It has been suggested by Wagner (1955) that the cytopathic changes in the cells may be due to a toxin.

Attempts to apply the plaque technique to investigations of influenza virus have not been completely successful. Granoff (1955a) failed to obtain plaques with chick fibroblasts and epithelial cells, although more recently, positive results have been obtained with chick embryo lung cells (Ledinko, 1955) and with calf kidney cells (Zimmermann and Schäfer, 1959).

Cytopathic effect.

As has already been mentioned, it was not until comparatively recently that influenza virus was found to have a definite cytopathogenic effect in tissue culture (Pollard and Bussel, 1953); since that time, many reports of the changes

induced by this virus have appeared in the literature.

Fragments of chorio-allantoic membranes, stained with pyronin-methyl green, have been examined at various stages after infection with influenza virus (Bate, 1955). The first detectable changes were a deepening in pyronin staining and a greater granularity in its distribution within the cytoplasm; this continued until 48 hours after infection when there was a heavy mass of coarsely aggregated red-staining material containing RNA in the immediate perinuclear region. Cytoplasmic vacuolisation was also common and the nucleus showed a characteristic disintegration with the appearance of rounded masses which stained with methyl green or haematoxylin. This suggested the appearance of inclusion bodies but although inclusion bodies have been observed in the bronchial epithelium of the mouse after infection with various strains of influenza virus (Harford et al., 1955) such changes are not generally associated with infection with this virus.

The morphological alterations occurring in monkey kidney cells after infection with influenza virus have been described by Mogabgab et al. (1955) in the following sequence:

1) granularity of the cytoplasm with swelling and rounding of the involved cell;
2) increasing granularity and shrinking of the nucleus;
3) appearance of rounded cells, first at the edge of the cell sheet but later throughout;
4) pyknotic and fragmented cells with scattered debris.

In a more detailed study carried out on the effect of influenza A strains on calf kidney tissue cultures, Negroni and Tyrrell (1959) observed depressed mitosis and rounding or vacuolation of the cells. The cytoplasm of infected cells stained more deeply for RNA and the surface of some cells appeared to be "boiling". By means of the haemadsorption technique, the appearance of new virus could be detected some hours before cellular changes became visible; with a large dose (e.g. 1 haemagglutinating unit) the first changes were seen 18 to 24 hours later and the type of change varied with the strain of virus used. In all cases the cytopathic effect was associated with the production of new virus haemagglutinin. Similar results have been obtained by other workers and Wulff and Haas (1957) have mentioned heavy vacuolation of the cytoplasm in swine kidney cells, especially with the PR8 strain of virus.

On the basis of the findings described above, the cytopathic effect induced in cells by influenza virus can be classified in Enders' group 1. This includes: "rounding or assumption of irregular or bizarre outlines, granulation of the cytoplasm, swelling of the cell, nuclear swelling, pyknosis and fragmentation of the nuclear chromatin, cell death and disintegration" (Enders, 1954).

Acridine orange technique. The use of the acridine orange staining method, which differentiates between intracellular RNA and DNA, has provided some interesting results in influenza-

infected cells. In the normal cell, the nuclear chromatin, which contains DNA, emits greenish-yellow fluorescence, whereas the cytoplasm and nucleolus, which have an appreciable content of RNA, fluoresce flame red (Armstrong and Niven, 1957). However, when calf kidney cells were infected with influenza virus, considerable alterations were observed and have been described as follows by Niven (1959):

"Enlargement of the nucleus and of the nucleolus was accompanied by the development of a diffuse red colour that filled the nucleus, often obscuring all structures except the associated chromatin of the nucleoli. The sharp outline of the nuclear membrane became indistinct and acquired a fine beaded appearance suggestive of breaks in continuity. In the cytoplasm, an increase in intensity of RNA fluorescence frequently was followed by the development of intense perinuclear fluorescence or by the appearance of tufts of flame red colour extending from one or both nuclear poles." In these experiments the intranuclear RNA fluorescence could be removed by RNAase, thus demonstrating the accuracy of the technique. It seems significant that, with an RNA-containing virus such as influenza, the dominant change should occur in the RNA fraction, but as has been mentioned previously, there is no justification at the present time for assuming that the RNA detected by the acridine orange technique is viral RNA.

Fluorescent antibody technique. This technique was first used in investigations of influenza virus infections by Watson and Coons (1954), who studied the growth of the PR8 and Lee strains

in chick embryos. Frozen sections of embryos in various stages of infection were treated with fluorescein-labelled immune serum and the extent and brightness of the staining was correlated with increase in infectivity and appearance of haemagglutinins. Multiplication was characterised by a diffuse type of staining which was first detectable in the nuclei and later in the cytoplasm of susceptible cells. In a later investigation Liu (1955), using fluorescein-labelled antibody to study influenza infections in ferrets, was able to demonstrate similar nuclear fluorescence. By absorbing the antisera with antigens from different strains it was possible to demonstrate that the S antigen was responsible for such nuclear fluorescence. More recently, Deibel and Hotchin (1959) have applied the technique quantitatively to tissue cultures of chick embryo cells, the FL line of human amnion cells (Fogh and Lund, 1957) and calf kidney cells infected with the Asian and PR8 strains of influenza.

A different situation might be expected in the case of influenza-infected HeLa cells owing to the fact that these cells can only support an incomplete cycle of multiplication (Henle et al., 1955) and that the progeny contain less S antigen; however, nuclear fluorescence has been demonstrated in influenza-infected HeLa cells, indicating the multiplication of S antigen (Wheelock and Tamm, 1959). These workers have also carried out experiments demonstrating that the cells containing newly-made influenza or NDV particles are still able

to undergo mitosis and to divide. This finding is in agreement with that of Rubin and Temin (1958) who used the Rous sarcoma-chick fibroblast system; but is in conflict with the work of Marcus and Puck (1958) carried out with the NDV-HeLa cell system. The technique has also been applied to obtain information about the precise sites of growth of neurotropic strains of influenza A (Fraser et al., 1959). The results show that the neurotropic NWS virus multiplies in this host, whereas the Mel strain, although producing specific fluorescence, only undergoes an incomplete cycle of multiplication. Such an incomplete cycle appears to be analogous to that occurring in influenza-infected HeLa cells and may be due to the inability of the virus to escape from the cell.

Electron microscopy. Although nuclear changes can be detected in influenza-infected cells by various histological techniques, virus particles have so far not been observed in electron micrographs of infected cells and are only visible at the cell surface as spherical or filamentous particles (Murphy and Bang, 1952; Wyckoff, 1953; Morgan et al., 1956). It was, in fact, thought that the filaments and spheres projecting from the cell represented the only visible changes until late in infection (Murphy and Bang, 1952) but in a recent study by Morgan and Rose (1959) cellular damage was demonstrated. When dilute inocula were employed, only a few cells showed morphological evidence of damage, in spite of the fact that the virus

produced achieved full infectivity, whereas with undiluted inocula, more damaged cells were seen and more filamentous forms. Nuclear changes observed by Morgan and Rose included irregular condensation of chromatin interspersed with "peculiar clear areas". A characteristic nuclear change was shown by an Asian strain of influenza in the form of intra-nuclear aggregations of material. It is suggested that this and the nuclear changes observed with other strains may be related to the production of a viral component, presumably S antigen. This suggestion is well supported by evidence obtained using the fluorescent antibody technique, which demonstrated that S antigen multiplies in the nucleus of influenza-infected cells (Liu, 1955).

Newcastle disease virus.

Culture.

Since the original isolation from fowls of the causative agent of Newcastle disease (Doyle, 1927) many hosts have been used to cultivate this virus. These were at first mainly avian and the allantoic cavity of the chick embryo is still used for routine culture of the virus (Gordon, Birkeland and Dodd, 1952; Nadel and Eisenstark, 1955; Macpherson and Swain, 1956). The use of chick embryo tissue cultures has also been quite extensive (Bang, 1953; Fastier, 1954; Pereira and Gompels, 1954; Mason and Kaufman, 1955; Bang and Warwick, 1957; Goldwasser and Kohn, 1957; Rubin et al., 1957; Brandt,

1958) and the production of plaques has been successfully demonstrated in chick embryo monolayers, providing an accurate titration method (Bower, 1957). These methods still involve the use and maintenance of eggs from a known disease-free source and, as it was obviously advantageous to develop a more labour-saving technique, the growth and titration of the virus in other tissues has been investigated.

The multiplication of NDV in HeLa cells and the resultant cytopathic changes have been described briefly by Tyrrell (1955) and by Bankowski and Hyde (1957). Tyrrell reported that, with the virulent Hickman strain, degeneration of the cells was obvious 24 hours after infective virus had reached its peak in the medium. Haemagglutinins were, however, not detected. Bankowski and Hyde found that, with the virulent GB strain and the avirulent TC strain, only irregular microscopic changes were visible in the cells during early passage of the virus. It was necessary to pass the virus many times before regular and marked cytopathogenicity could be obtained. In contrast to influenza virus (Henle et al., 1955) NDV appears to undergo a complete cycle of multiplication in HeLa cells. Other tissues which support the growth of NDV include monkey kidney cells (Chanock, 1955) and bovine kidney cells (Bankowski and Hyde, 1957).

A series of experiments was carried out to determine the effect of NDV on tumour cells and although the virus was found to inhibit the growth of Sarcoma 180 in mice, no evidence

was obtained for the multiplication of the virus in these cells (Moore and Diamond, 1952, 1953). Similar results were demonstrated with influenza virus and it was thought that this inhibition might be related to the haemagglutinating property of these viruses or to a mechanism akin to haemolysis (Burnet, 1949). This explanation was not compatible with the findings of Ginsberg (1951) and Davenport (1952) where the data suggested that the Newcastle virus damaged the cells from an intracellular position. To investigate these differences, further experiments were carried out on the NDV-Ehrlich ascites tumour cell system (Prince and Ginsberg, 1957 a, b). It was found that, although the death of the cells was not accompanied by demonstrable multiplication of infectious, haemagglutinating, or complement-fixing particles, the mechanism was similar to that occurring in a viral infection and did not result solely as a result of the reaction of the virus with the tumour cell surface. This interpretation was further supported by an electron microscope study (Adams and Prince, 1957) and a study employing the fluorescent technique (Prince and Ginsberg, 1957 c) in which an "incomplete" intracellular viral antigen was detected. Since these experiments were carried out, NDV has been adapted to growth in Ehrlich ascites tumour cells (Flanagan et al., 1955; Moore and Diamond, 1956).

Cytopathic effect.

Although the destructive effect of NDV in tissue cultures has been employed in titrating techniques (Fastier, 1954; Mason and Kaufman, 1955; Goldwasser and Kohn, 1957) descriptions of the morphological alterations occurring in infected cells are relatively few. Chanock (1955) has provided quite a detailed report of the sequence of events occurring in NDV-infected monkey kidney cells; the lesions began as discrete foci which gradually increased in size; dense granularity occurred in the cytoplasm and was followed by swelling of the cell, the cells then broke away from the epithelial sheet and disintegrated. Syncytial masses could also be distinguished. Usually 10 to 50 per cent. of the cells were left unaffected (see Marcus and Puck, 1958). A detailed study was carried out by Bang and Warwick (1957) on the effect of an avirulent and a virulent strain of NDV on cells in tissue culture; although the strains differed in their effects on chick embryo fibroblasts, macrophages and Walker rat carcinoma, the nature of the changes is not discussed.

It can be seen from Chanock's description that the cytopathic effect induced by NDV in monkey kidney cells may be classified in Enders' group lll, i.e. "formation of giant cells or coalescence of cells into syncytial masses". The virus thereby differs from influenza virus which induces a group 1 effect in monkey kidney cells and other tissue cultures but resembles mumps virus, which may also be classified in group lll.

The effect produced by mumps virus in HeLa cells was termed "cytolytic" by Henle et al. (1954) and it was suggested by these workers that such an effect may be related to the haemolytic property of the virus. NDV, like mumps, also possesses a haemolysin and as influenza does not, it seems conceivable that this may be responsible for the difference in cytopathic effects.

Electron microscopy. An electron microscopic study of the development of NDV in chick embryo cells was carried out by Bang (1953). Characteristic virus "filaments" were found to occur at the surface of the cell before morphological changes were detected within the cell. These filaments occurred as long delicate projections from the surface of the individual epithelial cell and apparently contained formed and partially formed virus particles. Fibroblasts were usually destroyed releasing scattered spherical virus particles. An electron microscope study of chicken macrophages infected with NDV has since been carried out (Hotz and Bang, 1957) but, in spite of considerable improvements available in technique, it was still impossible to distinguish the intracellular development of the virus. However, although the virus was not seen on the cell surface in the initial stages of infection, it could be seen in the ingested fluid droplets within the cell. Destruction of the entire cell surface was apparent five hours after infection but no specific lesions could be detected.

The development of filamentous projections and the inability to detect an intracellular form of the virus seem to be characteristic of the myxovirus group; besides occurring with influenza virus (Murphy and Bang, 1952; Wyckoff, 1953; Morgan et al., 1956) such findings have been obtained with mumps virus (Bang and Isaacs, 1957) and fowl plague virus (Hotz and Schäfer, 1957). Variations may occur, however, with adaptation and virulence and these have been summarised by Bang and Isaacs (1957).

Fluorescent antibody technique. Apart from the work of Prince and Ginsberg (1957 c) previously mentioned, in which an "incomplete" intracellular viral antigen was detected in the cytoplasm of Ehrlich ascites tumour cells, the fluorescent antibody technique has not been greatly used in investigating the growth cycle of NDV. Burnstein and Bang (1958) employed fluorescent antibody in a study of the pathogenesis of NDV in the chick and were able to demonstrate virus in the same areas in which lesions were present. It was later shown by Wheelock and Tamm (1959) that the viral antigen first appeared in the cytoplasm of NDV-infected HeLa cells as small foci of fluorescent staining; these foci increased in size until the cytoplasm appeared full of clumps of antigen. In contrast to influenza, however, no fluorescence could be detected in the nucleus. The multiplication of mumps virus has also been found to take place in multiple foci in the cytoplasm of cells (Watson, 1952); this lends further support to the work of

Ginsberg et al. (1948) suggesting that the mechanism of growth of the viruses of influenza and mumps is different.

The member of the myxovirus group most studied by the fluorescent antibody technique up to the present time is the fowl plague virus and the results obtained from these extensive investigations have shed considerable light on the mode of multiplication of this virus. Using fluorescent antibodies against the two antigenic components of fowl plague virus, the g-antigen and the haemagglutinin, Breitenfeld and Schäfer (1957) followed the development of these antigens in chick embryo fibroblasts; the g-antigen was first detected in the region of the nucleus, later it could also be found in the cytoplasm. The haemagglutinin was detected slightly later than the g-antigen and was distributed throughout the cell; it eventually accumulated in the peripheral region of the cell and could also be demonstrated in fine filaments which protruded from the cell margin. It seems possible that such filaments are identical to those observed by Hotz and Schäfer (1955) in electron micrographs of fowl plague-infected cells.

A further study with fowl plague virus was carried out in tissue cultures of chicken macrophages and giant cells and again showed the g-antigen developing in the nucleus, and the haemagglutinin, as before, was detected later than the g-antigen and was found only in the cytoplasm (Franklin, 1958). This evidence for growth of the virus within the nucleus of the infected cell supports a similar finding by Flewett and Challice

(1951) in investigations using phase contrast and electron microscopy. These workers also reported the presence of filaments in the cytoplasm which, they suggested, may have emerged from the nucleus into the cytoplasm.

An abortive or incomplete infection of Earle's L-cells has been studied by the fluorescent antibody technique (Franklin and Breitenfeld, 1959); g-antigen was produced in the nucleus but the normal release of this antigen into the cytoplasm and its subsequent union with haemagglutinin to form infectious particles was prevented. This may be analogous to the situation which occurs in HeLa cells infected with influenza virus (Henle et al., 1955) and in mouse brain infected with non-neurotropic strains of influenza (Fraser et al., 1959).

MULTIPLICATION OF THE MYXOVIRUSES.

The infection of a cell by a virus may be divided into three stages:

1) attachment of the virus particle to the host cell;
2) propagation of the virus within the cell;
3) release of the newly developed virus from the cell.

Attachment to the cell.

The myxoviruses attach themselves to the host cell by means of an affinity for the mucoprotein cell receptors. This affinity is generally considered to be due to the presence of an enzyme (Hirst, 1942 b, 1948) but it has been shown that cells which are no longer susceptible to enzyme action as a result of periodate treatment can still be infected (Fazekas de St. Groth & Graham, 1949). Bauer (1953) suggests that a non-enzymic affinity of virus subunits for mucoids is responsible for the reaction between virus and host cell.

After adsorption, the virus particles seem to be engulfed into the cell by a process similar to "viropexis", a word originally coined by Fazekas de St. Groth (1948) to describe the entry of influenza virus into an allantoic cell. The fact that macrophages in tissue culture engulf fluid droplets was originally reported by Lewis (1931) and this biological phenomenon was termed "pinocytosis" or drinking by cells. Since that time, "pinocytosis" has been found to be a general phenomenon among animal cells (Gey, 1955) and it seems very possible that, after the initial stages of virus adsorption,

entrance into the cell is part of this active process. This suggestion has received confirmation from electron micrographs of influenza-infected allantoic membranes (Flewett, 1953) in which it appeared as if the cytoplasm had "flowed over" the virus particles. In a more recent electron microscope study of chicken macrophages infected with NDV, Hotz and Bang (1957) observed virus particles in ingested fluid droplets within the host cells.

Intracellular multiplication.

A characteristic property of the intracellular multiplication of the smaller viruses is the occurrence of an eclipse phase; during this period, which may vary in length from 10 minutes in the case of bacteriophage (Ellis and Delbrück, 1939) to about 6 hours for strains of influenza A (Henle and Rosenberg, 1949), no increase in free infectious virus may be demonstrated. This indicates the occurrence of a reproductive cycle more complex than binary fission which is responsible for multiplication of bacteria and the psittacosis group of viral agents (Bedson and Gostling, 1954).

Influenza. Numerous analyses of the growth of the influenza viruses have been carried out, especially in the allantoic cavity of the chick embryo, where the allantoic fluid may be titrated at intervals for infectivity and haemagglutinating activity. A considerable advance was made in this direction when Henle et al. (1947) developed a method by which single

growth cycles could be isolated. These workers found that the single growth cycle of influenza consisted of the latent period of 5 - 6 hours, after which the maximum titre was soon reached; with influenza B the latent period was slightly longer. The rise in titre in one such step was about 60 - 80 fold and somewhat less for influenza B. In further experiments (Henle and Henle, 1949), the complement-fixing titre and haemagglutinating titre were also measured, thereby making possible the detection of S antigen and haemagglutinin. It was found that while the infectious titre of the allantoic fluid was still very low, an increase in S antigen occurred in membrane extracts 3 hours after infection and in the allantoic fluid 4 - 5 hours after infection; a rise in haemagglutinin occurred slightly later. In more recent experiments Liu and Henle (1951) reported that haemagglutinin was demonstrable as early as complement-fixing antigen. It has been suggested that the soluble antigen and haemagglutinin precede the infectious virus and are immature stages in the synthesis of new virus.

Similar studies of the multiplication of influenza virus in the chick embryo were carried out by Hoyle (1948, 1950). He showed that no virus could be detected in the cells by any method within the first 2 hours after infection; complement-fixing antigen was first detectable 2 hours after infection and by 3 hours haemagglutinin was also measurable. The infectivity titre did not rise until 5 - 6 hours after infection. These results show considerable agreement with those obtained by

Henle and co-workers although they reported the concomitant production of complement-fixing antigen and haemagglutinin (Liu and Henle, 1951).

Newcastle disease. The growth of NDV in the chick embryo has been studied by Gordon et al. (1952). These authors confirmed the presence of a 4-hour latent period and suggested that it was due to intracellular development of virus particles. Haemagglutinin was detected after infective virus indicating that the haemagglutinin unit resulted from a maturation of the infectious particle. These results were contradicted by Nadel and Eisenstark (1955) who reported the occurrence of allantoic fluids with moderate haemagglutinin titres and no detectable infectious titre, but other workers have failed to observe such a phenomenon (e.g. Granoff, 1955 b). A recent study of the growth of the virus in cultures of chick embryo lung epithelium was carried out by Rubin et al. (1957) by means of the plaque technique. No evidence was obtained for the presence of infectious virus at any other place than the cell surface. This supported the findings obtained in electron microscope studies (Hotz and Bang, 1957) where virus particles could not be detected within the cells.

Liberation of new virus.

The release of virus from the cell has been studied for three members of the myxovirus group; influenza A (Murphy and Bang; Wyckoff, 1953); Newcastle disease (Bang, 1953) and fowl plague (Hotz and Schäfer, 1955) and it appears that this stage

in virus replication is dependent on the presence of virus filaments. The filament of influenza is now thought to be formed as a virus-containing extrusion of a pathological cell (Bang and Isaacs, 1957) and each infective particle is formed as a budding off of this filament at which stage host protein is probably incorporated (Knight, 1946; Smith, Belyavin and Sheffield, 1955; Frisch-Niggemeyer, 1959). Such an explanation is in agreement with the findings that the host cell wall plays an essential role in the achieving of full infectivity by the virus particle (Hoyle, 1950; Cairns and Mason, 1953; Burnet, 1957) and would explain the fact that virus particles are only detectable at the cell surface (Murphy and Bang, 1952; Flewett, 1953).

Suggested mechanisms.

One of the most discussed and contradicted theories put forward to explain the multiplication of the influenza viruses is that of Hoyle (1950). This concept was based on the findings, described above, that in influenza infections a latent period occurs during which complement-fixing antigen and haemagglutinin become measurable; this precedes the appearance of infectious virus. Hoyle (1953) has summarised the events occurring during the reproduction of influenza virus as follows:

"The agglutinin is responsible for the initial union with the cell. On entry into the cell the lipid envelope fuses with the wall of the host cell, the elementary body is disintegrated

and the smaller units enter the cell. The soluble antigen is a self-replicating ribonucleoprotein which multiplies in the cell. At a later stage agglutinin is formed from the soluble antigen, the particular type of agglutinin being conditioned by the presence of agglutinin from the original inoculum,which acts as a pattern. The agglutinin and soluble antigen tend to aggregate and the complex possesses enzymic activity and is able to destroy the mucoprotein of the cell wall and protrusions develop which contain aggregating virus protein. The protrusions break away from the cell and ultimately form elementary bodies as a result of loss of water and continuing internal aggregation of the virus units."

There is now considerable evidence in favour of Hoyle's hypothesis including the apparent incorporation of host protein (Smith et al., 1955) and host lipid (Frommhagen et al., 1958) into the virus particle. Furthermore, it has been found that influenza virus filaments are ruptured by the same agents which rupture cell surfaces (Burnet and Lind, 1957). The genetically based theory of Burnet and Lind (1951), which regards the virus particle as a number of genetic units replicating separately and aggregating to form infectious virus, is also compatible with such findings.

One of the most contrary views is taken by Fulton (1953) who considers that viruses multiply by binary fission. He reported the appearance of specific complement-fixing antigen associated with the elementary body at the same time as the S antigen (Fulton, 1949). But the main theories postulated to

explain the reproduction of the influenza group of viruses follow the general pattern outlined by Luria (1953): the virus particle, after infecting the host cell, is transformed into non-infectious materials which reproduce inside the cells; new virus particles are formed by steps, being preceded by the formation of different elements which eventually become incorporated into infectious virus. Such an explanation is well supported by growth curve studies (Hoyle, 1948; Henle and Henle, 1949; Burnet, 1951 a, b; Burnet and Lind, 1951 a, b), by electron microscope studies, where it has been shown that virus particles as such cannot be detected within the cells (Murphy and Bang, 1952; Morgan et al., 1956), by chemical studies (Knight, 1946; Bauer, 1953; Smith et al., 1955) and by fluorescent studies (Watson and Coons, 1954; Liu, 1955).

The general pattern of multiplication of the other myxoviruses may be similar to that described for influenza; fowl plague, especially, has been shown to have an almost identical growth cycle (Schäfer, 1959). On the other hand, the fluorescent antibody technique has detected differences in the site of multiplication in the case of NDV and mumps; the first sign of antigen appeared in the cytoplasm of NDV or mumps infected cells (Watson, 1952; Wheelock and Tamm, 1959), whereas with influenza and fowl plague, the first demonstrable fluorescence was observed in the nucleus (Watson and Coons, 1954; Breitenfeld and Schäfer, 1957).

Incomplete virus.

Influenza.

In growth studies of influenza virus in the chick embryo and in other host systems, it has been found that infectivity and haemagglutinating activity develop in parallel and therefore, in freshly harvested virus suspensions, most of the particles can be assumed to possess both haemagglutinating and infective properties; in such suspensions the ratio of infective doses (ID) to haemagglutinating units (HA) has been found to be of the order of 10^6 (Henle and Henle, 1949; Hoyle, 1950; von Magnus, 1951a; Fazekas de St. Groth and Cairns, 1952). Under certain conditions, however, a considerably lower ID/HA ratio, e.g. 10^3 may be obtained due to the production of so-called incomplete virus particles which have haemagglutinating properties but which have a decreased infectivity. These incomplete particles may occur in various circumstances.

The von Magnus phenomenon. Although it had previously been observed that passage of undiluted influenza-infected fluid gave rise to virus with lower infectivity than that of the original inoculum (Henle and Chambers, 1941; Nigg et al., 1941), the phenomenon of incomplete virus formation was not described or fully investigated until some years later. While investigating the propagation of influenza virus in the chick embryo, von Magnus (1946) found that, during undiluted serial transfer of influenza virus in eggs, haemagglutinating titres of equal levels occurred in all passages but infectivity titres became

very low. To explain this phenomenon, he suggested that a non-infective variant of the PR8 strain had been formed, this variant possessing the same biological properties as the infectious virus with the exception of infectivity. When present in sufficient amount this virus interfered with the propagation of infective virus in mice and eggs, giving rise to suspensions of low infectivity but high haemagglutinating activity. The phenomenon was therefore thought to be due to "auto-interference".

After the preliminary observations, the formation of non-infectious influenza virus was investigated extensively by von Magnus (1951 a, b; 1952 a, b). The pattern of decreasing infectivity and constant haemagglutinating activity during undiluted serial passage was found to be highly reproducible and by the third passage the bulk of virus produced was "incomplete", only one out of 10,000 particles at most being fully active. von Magnus obtained experimental evidence that the divergences between the infectivity and haemagglutinating titres in these subcultures were due to the production of non-infectious haemagglutinins (NIHA); this NIHA he named incomplete virus, which he suggested represented an intermediate, immature form of the fully active virus which was not caused by extraviral substances (von Magnus, 1951, b; 1952, b). The incomplete form was only produced in appreciable amounts in embryos inoculated with large quantities of virus.

The experiments of von Magnus were confirmed by Fazekas de

St. Groth and Graham (1953, 1954 a) who found, however, that different strains of influenza virus differed widely in their capacity to yield incomplete offspring. These strains could be arranged in order of decreasing incomplete virus production to form a gradient which resembled closely the receptor gradient of Burnet et al. (1946). More recently, incomplete virus formation has been induced by relatively dilute inocula after treatment of the cell receptors with potassium periodate (Fazekas de St. Groth and Graham, 1954 b; 1955 a). Liu et al. (1956) have, however, described experiments which failed to indicate that loss of receptors influenced incomplete virus production.

The occurrence of incomplete influenza virus has been demonstrated in other host systems. Bernkopf (1950), working with the PR8 strain in de-embryonated eggs, reported the presence of non-infectious haemagglutinins incapable of self-propagation; the occurrence of this incomplete virus depended on the presence of large amounts of active virus in the medium. Further experiments with de-embryonated eggs were carried out by Daniels et al. (1952) and Burnet et al. (1955). A similar phenomenon was observed in mouse brain (Schlesinger, 1950) but it should be mentioned in this connection that the mouse brain, in contrast to the chick embryo, is generally considered unable to support the complete multiplication of non-neurotropic strains of influenza virus (Fraser, 1959). In agreement with

von Magnus, Schlesinger suggested that the incomplete virus in the mouse brain occurred as a result of the reproductive process rather than from viral degradation. The theory that incomplete virus was not a developmental stage in viral multiplication was put forward by Ginsberg (1954) who observed the phenomenon in mouse lungs. He suggested that the development of non-infectious haemagglutinin was correlated with and probably dependent on the host cell injury effected by the original virus inoculated.

Tissue culture observations of the phenomenon have not been so extensive. Daniels et al. (1952), in growth experiments in chick embryo tissue, observed that with concentrated inocula of influenza virus an increase in haemagglutinins occurred without an increase in infective virus. Similar evidence of incomplete virus formation was obtained in HeLa cells (Henle et al., 1955; Girardi et al., 1956), in chick embryo lung cells (Ledinko et al., 1957) and in chick embryo kidney cells (Mannweiler, 1959). In HeLa cells, as in the mouse brain, influenza virus cannot undergo a complete cycle of multiplication and although non-infectious haemagglutinins are produced as a result of infection with large inocula, this virus has been found to differ in interfering activity from that induced by undiluted passage in eggs (Paucker and Henle, 1958).

Other types of incomplete virus.

Non-infectious haemagglutinins are known to occur

normally in crude preparations of influenza virus (Friedewald and Pickels, 1944) and have also been demonstrated in the allantoic membrane of the chick embryo after inoculation with small amounts of virus (Granoff, 1955b). Apart from their lack of infectivity these particles resemble mature virus particles and may be termed intracellular non-infectious haemagglutinins; they are non-sedimentable and differ from the incomplete virus or extracellular non-infectious haemagglutinins previously described in their lack of interfering ability (Paucker and Henle, 1958).

Virus suspensions, partially inactivated by heat, have been found to resemble undiluted passage (UP) virus although they yield more infectious virus than the corresponding UP suspension (Paucker and Henle, 1955). Recently, however, differences between the two types have been observed in the electron microscope (Birch-Andersen and Paucker, 1959).

Another form of influenza virus thought to resemble incomplete virus is the filamentous form found chiefly in recently adapted strains (Chu et al., 1949; Murphy et al., 1950). It has been shown that ultrasonic treatment increased the haemagglutinating activity of the filament without a corresponding rise in infectivity (Donald and Isaacs, 1954 b) and electron micrographs showed a resemblance to incomplete virus in the lack of electron-dense centres (Morgan et al., 1956). Incomplete virus from undiluted passage has, however, been shown to be spherical (Werner and Schlesinger, 1954) and

it appears that the process of filament formation is independent of incomplete virus production. It is possible that the filamentous forms, like incomplete virus, may be lacking in RNA (Frisch-Niggemeyer, 1959) but as with all abnormal forms, it is difficult to obtain even partially pure preparations for chemical analysis.

Properties of incomplete virus.

In spite of being non-infective, incomplete virus resembles infectious virus in its ability to immunize (von Magnus, 1951 b) and to be adsorbed to and eluted from fowl erythrocytes (Donald and Isaacs, 1954 a; Werner and Schlesinger, 1954). Many differences between these two types of virus have, however, been observed by the use of physicochemical methods.

Sedimentation experiments have shown that preparations of low infectivity contain a major sedimenting component sedimenting at 400-675 S (Hanig and Bernkopf, 1950; Gard et al., 1952; Pye et al., 1956) while standard preparations have a comparatively homogeneous main component sedimenting at 620-800 S. In the electron microscope, non-infectious forms appear flatter and more heterogeneous than standard viruses; they are less dense and no internal details can be discerned (Werner and Schlesinger, 1954; Voss and Wengel, 1955; Pye et al., 1956; Birch-Andersen and Paucker, 1959). In virus particles subjected to heat inactivation some internal structure could be distinguished (Birch-Andersen and Paucker, 1959).

In chemical analyses, incomplete virus preparations have

been shown to have a higher lipid content (Uhler and Gard, 1954), a lower ribonucleic acid content (Ada and Perry, 1956) and less internal S antigen (Lief and Henle, 1956) than complete virus. The toxicity of non-infectious viruses for mice is less than that of standard viruses (Bernkopf, 1950; Manire, 1957). Interference by incomplete virus has been reported even after ultraviolet inactivation, but it has recently been found that when the internal S antigen drops below a certain level the interfering activity becomes weaker (Paucker and Henle, 1958). It is interesting to note in this connection that the haemagglutinating components separated from standard virus by ether and the incomplete virus from HeLa cells did not interfere.

Factors involved in incomplete virus formation.

Type of inoculum. Formation of incomplete virus has been considered by most workers to occur only after a large inoculum (Bernkopf, 1950; Schlesinger, 1950; von Magnus, 1952 b; Ginsberg, 1954), and it is therefore dependent on a high multiplicity of infection. In a quantitative survey, however, Cairns and Edney (1952) opposed these observations with evidence showing that the production of incomplete virus following large inocula is not due to infection of individual cells by too many particles since the phenomenon occurred when only 1 per cent. of the susceptible cells were infected. In a more recent analysis (Finter et al., 1955; Liu et al., 1956), it was reported that formation of incomplete virus results only

under conditions of multiple infection, i.e. when each cell is infected by more than one virus particle, and furthermore, that this multiple infection must be achieved within a short period of time.

There is agreement that the strain of virus used affects the amount of incomplete virus produced (Fazekas de St. Groth, 1953, 1954 a; von Magnus, 1954) but there has been some controversy over the importance of the composition of the inoculum. In some cases it appears that incomplete virus can be produced after inoculation with large amounts of infectious virus (Bernkopf, 1950; Schlesinger, 1950; Ginsberg, 1954) which suggests a toxic effect (Wagner, 1955), but in other cases the yield of incomplete virus is considerably increased by the presence of incomplete virus (von Magnus, 1952 b; Henle et al., 1956) or by heat-inactivated virus (Horsfall, 1954; von Magnus, 1954; Finter et al., 1955; Paucker and Henle, 1955) in the inoculum. It has even been suggested that some incomplete virus can replicate to produce non-infectious haemagglutinins and then loses its power to do so (Burnet et al., 1955).

Heat inactivation. It has been suggested that heat inactivation plays a significant role in the production of incomplete virus (Horsfall, 1954) and more recently Deichman (1957) has observed that the von Magnus phenomenon results from thermal inactivation of the inoculum or of virus produced in the early stages of infection. These results have not been supported in the literature (Fulton and Isaacs, 1954; Finter et al., 1955;

Paucker and Henle, 1955). Furthermore, in recent electron microscope studies of standard virus and different types of incomplete virus, heat-inactivated virus has been shown to differ from non-infectious virus produced during undiluted serial passage (Birch-Andersen and Paucker, 1959).

Suggested mechanisms.

The origin and mechanism of formation of incomplete virus is still not clear and many hypotheses have been put forward to explain this phenomenon. These opinions may be grouped as follows:

1. The original theory of von Magnus (1946), in which he suggested the production of a non-infective variant which interfered with the production of infective virus, was soon discarded. Fulton (1953) followed this up, however, by suggesting uniformly changed virulence. This was opposed by the findings of Fazekas de St. Groth (1955 b) who reported that virus yielded by the von Magnus phenomenon and standard virus do not differ in the slope of their infectivity curves.

2. The hypothesis of "autointerference" postulated by von Magnus (1951 b) has been well used in explaining the production of incomplete virus after large inocula. It is still considered probable that incomplete virus represents an intermediate stage in the normal reproduction of influenza which does not reach completion due to a blockade of some metabolic system of the host cell.

3. In opposition to the above explanation are Schlesinger (1953) and Ginsberg (1954), who suggest that incomplete virus is the product of abnormal virus-cell interaction. Likewise, Fraser (1959) proposes that a defect of production is involved in incomplete virus formation.

4. Other authors have suggested that incomplete virus may be a degradation product of standard virus (Horsfall, 1954; Deichman, 1957) but the striking differences between standard and incomplete viruses previously enumerated point against this opinion.

5. Although agreeing with von Magnus that non-infectious virus occurs owing to an upset in the normal multiplication of the virus, Fazekas de St. Groth and Graham (1953) do not support the "autointerference" explanation of the mechanism of incomplete virus formation. These workers attribute incomplete virus formation to a mechanism related to the cell receptors; they suggest (Fazekas de St. Groth and Graham, 1955 a) the involvement of a chemical grouping of the cell surface in the production of infectivity and describe the mechanism as follows: "during viropexis (Fazekas de St. Groth, 1948) a small area of the ectoplasm folds into the cell, taking with it the infecting particle. If this ingested area contains more than a certain defined minimum of substrate for this viral enzyme, that cell will yield infective particles at the end of the multiplication cycle; if not, only a fraction of the yield will be infective, the rest consisting of incomplete non-infective particles."

6. Hoyle (1953) suggests that the production of incomplete virus after infection of the cells with large inocula is due to premature disintegration of the cytoplasm of the host cell before the intracellular cycle is complete. The particles are not released in the usual way and are therefore unable to achieve full infectivity from passage through the cell wall.

7. In the opinion of Burnet (1955), degrees of incompleteness are important and he postulates two types of virus:

a) virus which can replicate to produce haemagglutinin in one or two generations and then loses its power to do so;

b) haemagglutinin which cannot replicate but which can enter cells and after a few hours induce interference against the multiplication of complete virus.

8. Lief and Henle (1956b) suggested that lack of incorporation of S antigen was responsible for the decreased infectivity of incomplete virus. On the other hand, Paucker et al. (1959) proposed, that the altered morphology of non-infectious virus may be due to the reduced incorporation of the haemagglutinating antigen; this is based on the findings that decreased incorporation of S antigen alone, which represents only 14 per cent. of the total weight of the infective particle (Frisch-Niggemeyer and Hoyle, 1956), could not alone account for the striking differences occurring in size and density of complete and incomplete virus (Birch-Andersen and Paucker, 1959).

At first sight these opinions appear somewhat divergent, but on further examination it may be seen that apart from the

suggestion that incomplete virus may be a degradation product of the inoculum or of newly formed virus all these hypotheses support the idea that incomplete virus is formed as a result of some upset in the metabolism of the host cell brought about by infection with a large inoculum.

The so-called abortive cycles of multiplication which occur in hosts unable to support a complete cycle may be the result of some abnormality occurring in the release of virus from the cell. This may possibly provide an explanation for the events occurring in HeLa cells (Henle et al., 1955) and in the mouse brain (Fraser et al., 1959).

Production of incomplete virus by other myxoviruses. Newcastle disease virus. When experiments similar to those carried out by von Magnus with influenza virus were applied to Newcastle disease virus, no evidence of incomplete forms could be obtained (Granoff, 1955b; Horsfall, 1956; Scott, 1958). On the other hand, the existence of an incomplete virus stage in the maturation cycle has been suggested by Nadel and Eisenstark (1955) but this has not received support from other workers (Gordon et al., 1952; Cairns and Mason, 1953).

Granoff (1955b) has, however, described the presence of a non-sedimentable, non-infectious, haemagglutinin of NDV, called S haemagglutinin, which could be demonstrated early in the infectious cycle but not prior to the development of infectious virus. This appears to represent a developmental

stage in virus reproduction and may be analogous to the haemagglutinin demonstrated in the allantoic membranes of chick embryos inoculated with small amounts of influenza virus.

Other references to NDV include that of Davenport (1952) who reported an incomplete cycle in the mouse lung where cellular injury occurred without multiplication of infective virus; previously Ginsberg (1951) had reported no evidence of non-infectious haemagglutinins in this host. In immunohistochemical studies on the interaction between Ehrlich ascites tumour cells and NDV, an intracellular viral antigen was detected by means of the fluorescent antibody technique (Prince and Ginsberg, 1957 c) but the appearance of this antigen could not be correlated with a rise in infective or haemagglutinating titre. In further experiments with this virus-cell system intracytoplasmic inclusions were detected, and in electron micrographs it was shown that these inclusions contained dense particles measuring 3 - 14 mμ in diameter; (Adams and Prince, 1957); these particles were identified as the newly synthesised incomplete virus which had been previously demonstrated by the fluorescent antibody technique. It is possible that this virus occurred as the result of a multiplication cycle similar to that occurring in influenza-infected HeLa cells and mouse brain.

Fowl plague virus. With fowl plague virus, as with NDV, units comparable to the incomplete forms of von Magnus could not be produced by repeated serial passage (Schäfer, 1959).

Haemagglutinating, non-infectious, incomplete viruses have, however, been found in the allantoic membranes of chick embryos infected with fowl plague virus. These incomplete forms were larger, flatter and more heterogeneous than active viruses and seem to resemble those isolated by Werner and Schlesinger (1954) from tissues infected with influenza virus although they could not be isolated from allantoic fluid. It has been suggested by Schäfer (1959) that the incomplete forms of fowl plague are microsomes of the infected cells.

When the growth cycle of fowl plague virus in Earle's L cells was investigated by means of the fluorescent antibody technique (Franklin and Breitenfeld, 1959) evidence for the synthesis of non-infectious haemagglutinins and S antigen was obtained but no infectious virus was produced. It is suggested by these workers that the normal release of S antigen into the cytoplasm and the subsequent union of S antigen with haemagglutinin to form infectious particles is prevented. Furthermore, they suggest that the non-infectious haemagglutinins found in these cells and in chick embryos seem to be similar to the non-infectious haemagglutinins found in tissues infected with influenza or NDV. These viruses are, however, distinct from the extracellular incomplete forms which are produced in large amounts when embryonated or de-embryonated eggs are infected with large doses of some strains of influenza virus.

Mumps virus. In the case of mumps virus it was found that when

the initial particle-cell ratio was 3 or more, large amounts of haemagglutinating but non-infective particles appeared in the allantoic fluid (Horsfall, 1956). This observation was not entirely confirmed by Cantell (1959) although in her experiments, a decrease in the infectivity/haemagglutinating ratio during serial undiluted passages resembling the von Magnus phenomenon was observed. This seemed to be due to the formation of incomplete virus. The data also suggest that an interfering material similar to "interferon" (Isaacs and Lindenmann, 1957) may be produced.

VIRAL INTERFERENCE.

An interesting feature of virus growth which should be mentioned in any review of virus multiplication is the phenomenon of interference. This has been defined as: "the action of a virus, either live or inactivated, on cells, as a result of which the cells are rendered unable to support fully the growth of immunologically related or unrelated viruses." (Isaacs, 1959 a).

As the definition implies, interference may occur between immunologically related viruses, e.g. between different strains of influenza virus (Andrewes, 1942) so-called homologous interference, between viruses of the same group, e.g. between influenza and NDV (Bang, 1949), between unrelated RNA-containing viruses, e.g. between influenza and Western equine incephalitis (Henle and Henle, 1945) or between completely unrelated viruses, e.g. between influenza and vaccinia (Depoux

and Isaacs, 1954). The majority of the experiments on interference have been carried out in the chick embryo but several reports of the phenomenon in tissue culture have also been published; these include interference between NDV and polio in monkey kidney cells (Chanock, 1955), between influenza and polio in monkey kidney cells (Wesslén et al., 1959) and between different types of polio virus in monkey kidney and HeLa cells (Drake, 1958).

Although live virus is known to interfere, much of the work on interference has been concerned with the use of virus which has been inactivated, e.g. by heat, ultraviolet light, formaldehyde or nitrogen mustard. Since the original demonstration by Henle and Henle (1943) that inactivated influenza virus interfered with the growth of influenza viruses in the chick embryo, many investigations on this type of interference have been carried out; a very thorough review of these studies has been published by Henle (1950).

The induction of the interference phenomenon is dependent on many factors. The importance of dosage and timing of the interfering and challenge inocula have been emphasised (Fazekas de St. Groth et al., 1952; Henle and Paucker, 1958). It has also been found that the presence of a certain amount of S antigen is necessary to induce interference (Paucker and Henle, 1958); incomplete virus preparations containing S antigen below a certain level will not interfere. So far, however, there is no direct proof that the soluble

antigen is the actual interfering agent.

Interference is thought to result from many causes. The fact that the phenomenon occurs between completely unrelated viruses indicates the early blocking of some common metabolic pathway. Baluda (1957), on the other hand, attributes interference by NDV to receptor destruction; such an explanation can, however, only apply to viruses which share cell receptors of the influenza group and furthermore, interference can be induced by virus in which the enzyme action has been destroyed (Isaacs and Edney, 1950). It has recently been suggested that the interfering activity of inactivated myxoviruses on a variety of unrelated viruses may be brought about by the production of a substance called "interferon".

Interferon

When heat-inactivated influenza virus was incubated with pieces of chick chorio-allantoic membrane, the production of an interfering substance distinct from the original heated virus was observed. This new interfering material was called "interferon" (Isaacs and Lindenmann, 1957). After the initial investigations with influenza virus, several other myxoviruses including the PR8 and Mel strains of influenza A, the Lee strain of influenza B, fowl plague and Newcastle disease virus were found to produce interferon of comparable activity following incubation with whole chorio-allantoic membrane. At the present time, according to Isaacs (1959 a), there is no

reason to believe that the production of interferon is restricted to a particular type of cell; besides being observed in the chick embryo it has been observed in calf kidney cells (Tyrrell, 1959) in monkey kidney cells and in HeLa cells.

A correspondence has been noted between degree of interference and the amount of interferon liberated (Lindenmann et al., 1957) and it has been found that excessive irradiation or heat inactivation lead to a reduction in the ability to produce interferon and to a corresponding reduction in interfering activity. Virus irradiated by ultraviolet light has been found to induce the most potent preparations of interferon (Burke and Isaacs, 1958 b). It was originally thought that interferon could not be produced by live virus (Burke and Isaacs, 1958 a) but in more recent experiments Tyrrell (1959) demonstrated interferon production as virus production was declining. Unirradiated incomplete virus will also produce good yields of interferon (Burke and Isaacs, 1958 a).

Properties of interferon.

Interferon differs in several ways from the virus from which it is prepared. Attempts to induce neutralising antibody have been unsuccessful and it is not significantly adsorbed by chicken red cells. Physicochemical properties so far obtained for interferon suggest that it is a macromolecular protein of considerably smaller size than the virus. It is

relatively stable at 2° C. and over the pH range 2-11 at this temperature. The stability at pH 2 is useful for distinguishing it from inactivated virus. It is sensitive to pepsin and trypsin but insensitive to treatment with deoxyribonuclease, ribonuclease or the receptor-destroying enzyme of Vibrio cholerae (RDE). It resists treatment with 0.001 M-sodium periodate, which is known to react with many carbohydrates but is destroyed by shaking with ether.

Other interfering agents.

A series of experiments has been carried out by Henle and co-workers (Henle et al., 1958) on persistent infections in tissue culture. A high degree of resistance to cytopathogenic viruses was observed in these cultures, especially to the virus of vesicular stomatitis; the presence of an interference phenomenon was suggested. However, when the virus-producing cells were transferred to fresh cultures, interference was rapidly established and could not be accounted for by the rise in infectious virus. The possibility that interferon was produced in this system was later supported by the finding that an interferon-like substance was produced in uninfected cultures on addition of ultraviolet inactivated myxoviruses (Henle et al., 1959). The substance resembled interferon in that it was not neutralised by specific antibodies, it was not adsorbed on to red cells and it was largely inactivated by trypsin. It seems possible that interferon was involved in the resistance shown by the persistently infected cultures (Isaacs and Burke, 1959).

Another substance similar to interferon and with an inhibitory effect on several different viruses has been obtained from primary human kidney and amnion cell cultures infected with chick embryo adapted type 2 polio virus (Ho and Enders, 1959). This viral inhibitory fluid (VIF) differs from interferon in some properties:

1) it is optimally produced with active virus;
2) it adsorbs poorly to cells;
3) it acts most effectively if it is continuously present in the system;
4) interferon does not appear to inhibit type 1 polio virus infection in amnion cultures.

Although the differences apparently existing between VIF and interferon may be resolved (Ho and Enders, 1959), these authors suggest the occurrence of several different substances accidentally produced during virus-cell interaction which may be inhibitory to viral infection.

Mechanism.

The mechanism by which these interfering agents act is still not known but it is suggested that interferon may act by deflecting the cell from synthesising a normal virus intermediate to synthesising more interferon. It is possible that interference may eventually be defined as an inhibition of the formation of viral nucleic acid (Isaacs, 1959; Isaacs and Burke, 1959).

Reasons for the present investigation.

Various aspects of the relationship between viruses and their host cells have been reviewed in the preceding pages. The myxoviruses received particular attention, and on examining the literature it was found that there exist differences between certain members of this group, for example between Newcastle disease virus and influenza virus. The main differences may be enumerated as follows:

(a) Type of cytopathic effect. Newcastle disease virus induces the formation of syncytial masses in tissue culture, whereas influenza produces a more generalised effect.

(b) Incomplete virus. Up to the present time, the production of incomplete virus, which is readily observed following large inocula of influenza virus, has not been demonstrated with Newcastle disease virus.

(c) Site of intracellular multiplication. Fluorescent antibody studies have shown that the influenza virus develops in the nucleus and in the cytoplasm of infected cells, whereas, with Newcastle disease virus, specific fluorescence has so far only been demonstrated in the cytoplasm.

With such problems in mind, it was decided to study the growth in tissue culture of these two members of the myxovirus group. Newcastle disease virus in HeLa cells and influenza A virus in monkey kidney, bovine embryo kidney and HeLa cells were employed as virus-cell systems. The proposed plan of study was (a) to investigate the effect of these viruses on their host cells by means of various techniques and to correlate this effect

with the production of new virus; (b) to study cell survival following virus infection; and (c) to investigate the possibility of incomplete virus formation. In addition, the development of influenza virus was examined by means of the fluorescent antibody technique employing specific sera prepared against the soluble (S) and viral (V) antigens.

The work described here has, through force of circumstance, been carried out in two parts. The first part, on the growth of Newcastle disease virus in HeLa cells, was completed in the Bacteriology Department, University of Edinburgh, whereas the second part, which concerns the behaviour of influenza A virus in various tissue culture systems, was carried out in the State Serum Institute, Copenhagen, Denmark.

MATERIALS AND METHODS

Part I.

NEWCASTLE DISEASE VIRUS IN HELA CELLS.

Virus strains.

The virulent Hertfordshire (Herts) and California (Cal) strains and the avirulent Blacksburg (B.1) strain of Newcastle disease virus (NDV) were cultivated in the allantoic cavity of the chick embryo as described by Macpherson and Swain (1956). Infective allantoic fluids were stored at -20° C.

Tissue culture.

HeLa cells. These cells were obtained from Dr. Tomlinson, P.H.L.S., County Hall, Westminster, and monolayers of the cells were prepared from stock cultures dispersed by means of "Versene" (Disodium ethylenediamine tetra-acetic acid). The cell suspension prepared in this way was diluted to approximately 15,000 cells/ml. in standard tissue culture medium and was distributed in 0.5 ml. amounts into Pyrex test-tubes. The tubes were held stationary for 48 hours at 36° C., after which the medium was replaced by 0.9 ml. fresh medium. Incubation was continued for a further 4 - 5 days in a rotating drum and prior to the introduction of virus the culture fluid was replaced by 0.9 ml. fresh medium.

Standard medium. The medium used throughout these experiments consisted of 4 per cent. lactalbumin hydrolysate, 10 per cent. rabbit or calf serum and 75 per cent. Hanks' balanced salt solution to which had been added 100 units penicillin, 100 µg. streptomycin and 100 units mycostatin per ml. The infectivity

titre of the NDV allantoic fluid was not affected when the rabbit serum was replaced by calf serum.

Cell counts were carried out as follows: the medium was removed and the cell sheet washed twice with calcium and magnesium-free phosphate buffered saline. A sterile 1 : 5000 dilution of Versene in buffered saline was added to each tube in 1 ml. amounts and the tubes were incubated in the roller drum for 20 - 30 minutes. Counts were made in a Fuchs-Rosenthal haemocytometer.

Titration methods.

Fluids for titration were harvested and centrifuged at 500 r.p.m. for 5 minutes to remove cell debris. If possible, fluids were titrated immediately or after storage overnight at 4° C. On occasion it was necessary to titrate after storage at -60° C. but in such cases the haemagglutinating activity was measured before freezing.

Infectivity in HeLa cells. Serial tenfold dilutions were prepared in Hanks' solution and 0.1 ml. amounts of the appropriate dilutions were added to triplicate HeLa cell cultures. The tubes were incubated at 36° C. in the roller incubator and were examined daily for 5 days. The TCD_{50} (tissue culture dose) was calculated by the method of Reed and Muench (1938) from the number of tubes showing cytopathic changes.

Infectivity in eggs. Titrations were carried out by inoculating 0.1 ml. amounts of serial tenfold dilutions into 9 or 10-day old chick embryos by the allantoic route. Five eggs were used for each dilution. They were incubated at 36° C. and were candled daily; those dead at 24 hours were discarded. At 48 hours dead embryos were chilled at 4° C. and after 72 hours all embryos were killed and the allantoic fluids tested for haemagglutination. The EID_{50} (Egg infective dose) was calculated by the method of Reed and Muench (1938) from the eggs showing positive haemagglutination. Throughout the test, unless otherwise stated, the TCD_{50} and EID_{50} are expressed as the negative logarithm per 0.1 ml.

Haemagglutination tests. Serial twofold dilutions of the fluids to be tested were made in 0.25 ml. of calcium saline prepared from normal saline plus 0.1 per cent. calcium chloride, 0.1230 per cent. boric acid and 0.0052 per cent. sodium borate. Washed fowl red cells in 0.5 per cent. suspension were added in 0.25 ml. amounts and the tests were read after 20 - 30 minutes at 20° C. The haemagglutination (HA) titre was taken as the highest dilution showing complete agglutination.

Antiserum.

Immune serum to the Herts strain of NDV was prepared in the rabbit. Two intravenous inoculations of 1.0 ml. of infected allantoic fluid, spaced at an interval of 14 days,

were given. In neutralisation tests a constant dose of virus of known titre (i.e. 10^6 TCD_{50}/ml.) was added to equal quantities of tenfold serial dilutions of antiserum. The mixtures were left for an hour at 20° C., after which they were added to HeLa cell cultures in 0.1 ml. amounts. The tubes were examined daily for degenerative changes and the titre of the antiserum was taken as the highest dilution completely neutralising 10^6 TCD_{50} of virus suspension.

Staining technique.

Cells to be examined in this way were grown on coverslips and slivers in stationary Pyrex tubes and were infected with virus.

At timed intervals the strips were removed from the tubes, washed in balanced Hanks' solution and fixed for 30 minutes in Schaudinn's solution. After rinsing in distilled water they were left overnight in 1 : 10 Giemsa's stain. Differentiation was carried out with absolute alcohol.

Time lapse cinematography.

A slide perfusion chamber was devised for use with interference microscopy (Constable and Moffat, 1958) and was found to fulfil the optical requirements associated with this type of work.

Construction of chamber. The body of the chamber was made from a thin glass slide 3 in. x 1 in. x 1 mm., a hole approximately $\frac{1}{2}$ in. in diameter being cut in the centre by means of a

piece of copper tubing held in a vertical bench drill. No. 1 coverglasses, 0.18 mm. in thickness, formed the walls above and below. At each side of the perforation, a short groove was cut in the glass, parallel to the long axis of the slide by means of a carborundum wheel in a dental drill. These grooves were not deeper than was necessary to accommodate a stainless steel needle no. 20. At this stage all components were cleaned to the required standards for tissue culture.

To assemble the chamber, one coverglass was cemented over the hole in the slide, on the surface of which there were no grooves; two hypodermic needles were bent into the shape of a crank and were cemented into the grooves so that the points projected slightly into the chamber. Additional support was given in the form of small wooden blocks under the needle fitting to facilitate the attachment of syringes or tubing. Finally, a coverglass was cemented in place close to the chamber, the only access to which was through the needles (Fig. 3).

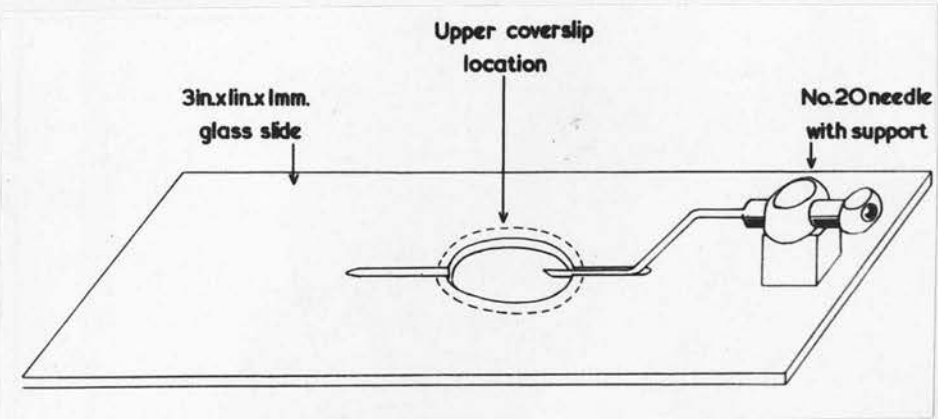

Figure 3.
Plan showing construction of culture chamber. (Constable and Moffat, 1958).

A satisfactory cement was found to be "Araldite", a two component epoxy resin obtainable from Aero Research Ltd., Duxford, Cambridge. At room temperature this adhesive takes at least 12 hours to set, but by raising the temperature this period can be reduced to as little as 30 minutes.

Sterilisation. The complete chamber was sterilised in the hot air oven at 160° C. for 1½ hours when the cement was found to be extremely hard and black.

Preparation of cultures. After sterilisation, the chamber was filled with a cell suspension containing approximately 100,000 cells/ml. in an appropriate medium. This was injected from a syringe directly attached to one of the needle fittings, and by tilting the slide all air could be excluded. After removal of the syringe, it was necessary to seal the needle ends with small plugs of cotton wool to prevent blockage by dried medium. A short period of incubation at 37° C. was required to allow the cells to settle on the upper coverglass, after which the chamber was turned over and was ready for observation in the microscope incubator.

Filming. The cells could be studied in situ and when a suitable field was found, either new medium or virus could be introduced, prior to filming, by means of a suitably geared motor-driven syringe which drew fluid through the chamber from a reservoir within the incubator. The actual filming was carried

out by the Photomicrography Unit (Director Mr. T.C. Dodds) in the University of Edinburgh. The film presented here was made on Agfacolor film with a pulse of 30 seconds.

Part II

INFLUENZA VIRUS IN MONKEY KIDNEY CELLS, BOVINE EMBRYO KIDNEY CELLS AND HELA CELLS.

Virus strains.

The PR8 strain of influenza virus used in this study was originally supplied by Dr. R.E. Shope, Princeton, N.J. It had previously been passed only in ferrets and mice (146 passages) and was established in chick embryos after 80 further mouse lung passages in the influenza department at the State Serum Institute, Copenhagen. When the present experiments were carried out the virus had undergone approximately 100 passages in the allantoic cavity of the chick embryo. Undiluted allantoic fluid had an approximate titre in eggs of 10^{10} EID_{50}/ml. and a haemagglutinating titre of 4 log HA units per ml.

The WS and Mel strains were obtained through the kindness of Dr. D.A.J. Tyrrell, Common Cold Research Unit, Salisbury, England, in August 1959. These strains had previously undergone passage in the allantoic cavity of the chick embryo and they were further passed twice in this host as a preliminary to the experiments described here. The strains had an infectivity titre of approximately 10^{10} EID_{50}/ml. and could be differentiated by the haemagglutination-inhibition test.

The Asian strain was an influenza A_2 strain, A-Denmark

18/57, kindly supplied by Dr. K. Birkum Petersen, State Serum Institute, Copenhagen. This strain was isolated in monkey kidney cultures and had been passed five times in the chick embryo before use in these experiments. The approximate titre of undiluted allantoic fluid was 10^9 EID_{50}/ml.

These strains were cultivated in the allantoic cavity of the chick embryo as described below under "standard virus". Infective allantoic fluids were stored overnight at -4° C. or for longer periods of time at -60° C.

Egg passages.

"Standard virus". The virus used in these experiments had undergone "standard" passage in eggs, i.e. passage in 11-day chick embryos of 0.2 ml. of allantoic fluid virus diluted 10^{-6}. After inoculation, the embryos were incubated for 42 hours at 35.5° C. The freshly harvested allantoic fluids were pooled and titrated and had an average titre in eggs of 10^{10} EID_{50}/ml.

"Undiluted passage virus" (UP virus). Serial passages of undiluted virus were initiated with freshly passaged standard virus. 11-day embryos were inoculated with 0.5 ml. of undiluted virus suspension and the allantoic fluids were harvested after incubation at 35.5° C. for 18 hours. They were then pooled and titrated and passed undiluted on the day of harvest. The successive subcultures have been termed the first, second, third, etc., passage of undiluted virus = UP1, UP2, UP3, etc. These

fluids may be referred to as EUP (egg undiluted passage) to distinguish them from virus which has undergone serial passage in tissue culture (TCUP virus).

Tissue cultures.

Monkey kidney cultures. These were prepared by a modification of Youngner's method (von Magnus et al., 1955). After removing the capsule, ~~the cortex and~~ most of the medulla ~~were~~ was removed from the cortex and minced with scissors and trypsinised for 5 minute periods at 37° C. The resulting cell suspension was centrifuged and re-suspended in Hanks' solution containing 0.5 per cent. lactalbumin hydrolysate, 2 per cent. calf serum, and 7.5 per cent. 1·4% sodium bicarbonate; 1.5 ml. penicillin + streptomycin solution per l. was included. This was the stock solution and 50 ml. of this was used per kidney for re-suspension. The cell suspension was then placed on the magnetic stirrer for a further 10 minutes and 2 ml. was removed for cell counting and sterility tests. Tubes were seeded with 1.2 ml. of the cell suspension which had been diluted to contain 200,000 cells/ml. The cultures were used for experimental purposes when 4 days' old and before inoculation the medium was replaced with Parker's medium 199.

Bovine embryo kidney cultures. Bovine embryo kidneys were treated by essentially the same method as that described above for monkey kidneys except that trypsinisation was carried out at 4° C. for 18 hours.

HeLa cells. This strain was originally obtained from Dr. Wilton Earle, National Institutes of Health, Bethesda, Maryland. The cells were propagated in a growth medium consisting of Earle's balanced salt solution, 0.06 per cent. yeast extract, 0.18 per cent. lactalbumin hydrolysate and 20 per cent. horse serum. Stock cultures, maintained in Roux bottles, were treated with Versene to obtain cell suspensions.

Titration methods.

Infectivity in monkey kidney cells. These titrations were carried out in the same way as the titration of NDV in HeLa cells. The 50 per cent. end-point was, however, calculated by the method of Kärber (1931) both for the TCD_{50} and for the EID_{50} which is described below.

Infectivity in eggs. Egg titrations were carried out according to the method of Hirst (1942 a). Serial tenfold dilutions of the virus suspensions in buffered saline were prepared and groups of 5 11-day old chick embryos were inoculated with 0.2 ml. of the various dilutions. After incubation at 35° C. for 72 hours, the eggs were chilled and the individual allantoic fluids were harvested and tested separately for the presence of haemagglutinins. Positive haemagglutination served as a criterion of infection and was used in calculating the 50 per cent. endpoint. Titres are expressed as the number of egg infective doses (EID_{50}) per ml. of undiluted test material.

Haemagglutination titrations. The pattern method described by Salk (1944) was used with minor modifications. For each titration two different serial twofold dilutions of the test material were prepared, each dilution in the one series being the geometrical mean of the two adjacent dilutions in the other. To each tube was added 0.5 ml. of a 0.25 per cent. suspension of red cells and the tubes were incubated overnight at 4° C. The number of haemagglutination (HA) units per ml. is expressed as the reciprocal of the final dilution of virus which agglutinated 50 per cent. of the red cells added.

Antisera. (see 81a)

Pure anti-S and anti-V sera were prepared in guinea-pigs using the method outlined by Lief, Fabiyi and Henle (Lief et al., 1958; Fabiyi et al., 1958). In chessboard complement-fixation tests, starting with 1 : 4 dilutions of both antigens and antisera, there was no cross-reaction between S antigen and anti-V serum. Vice versa, there was no crossing between V antigen and anti-S serum. The homologous titre of the anti-S serum employed was 1 : 256 and that of the anti-V serum was 1 : 181.

Whole antiserum, i.e. antiserum directed against both S and V antigen, was produced in guinea-pigs by intraperitoneal injections of an elementary body suspension obtained by one cycle of adsorption on to and elution from chick red blood cells, using the same immunising procedure as that employed for the production of specific anti-V serum. The specific titres of this serum were: anti-S 1 : 256 and anti-V 1 : 191.

Antiserum

Antiserum to the soluble (S) antigen of influenza virus was prepared in guinea-pigs (Lief et al. 1958). An intranasal inoculation of active virus was given initially and was followed in two weeks by a booster intra-abdominal injection of heterologous homotypic S antigen (Lief and Henle, 1956c).

Antiserum to the viral (V) antigen was prepared from the haemagglutinin fraction derived from elementary bodies treated with ether to remove all external and internal S antigen (Lief and Henle, 1956c). All preparations were irradiated by ultraviolet light. The serum was prepared in guinea-pigs by two intra-abdominal injections of vaccine given 5-7 days apart followed by a booster dose three weeks later (Fabiyi et al.1958).

In chessboard complement-fixation tests, starting with 1:4 dilutions of both antigens and antisera, there was no cross-reaction between S antigen and anti-V serum. Vice versa, there was no crossing between V antigen and anti-S serum. The homologous titre of the anti-S serum employed was 1:256 and that of the anti-V serum was 1:181.

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Complement-fixation tests were carried out according to the method of Fulton and Dumbell (1949) in the modification of Svedmyr et al. (1952).

Staining techniques.

Cells to be examined by staining techniques were cultivated in Leighton tubes (Pyrex test-tubes with a flat-sided well measuring 40 x 12 mm.). Coverslips (11 x 22 mm.) were placed in the well and the cell suspensions were added in the usual way. The cultures were used after approximately 5 days' growth when a sheet had been formed. Details of virus inoculation are given in the appropriate experiment.

Haematoxylin and eosin. Coverslip preparations washed in Parker's medium were fixed in methyl alcohol for 5 minutes, rinsed in 90 per cent. alcohol and stored in 70 per cent. alcohol. Staining was carried out by treating the preparations in haematoxylin for 10 minutes, differentiating in 1 per cent. HCl in alcohol for 1 second, staining in 1 per cent. eosin for 1 minute and finally differentiating in alcohol.

Acridine orange. Fixation was identical to that described above. Most of the acridine orange staining was carried out by Dr. Janet Niven, Mill Hill, London, N.W.7. Acridine orange C.I. no. 788 was used in an HCl acetate buffer and the preparations were examined by fluorescence microscopy using the blue-violet emission (about 4,500 Å) from a high pressure mercury vapour lamp.

Fluorescent antibody staining technique.

Preparation of rabbit anti-guinea-pig gamma globulin.

Guinea-pig gamma globulin was prepared by Dr. A. Hansen, State Serum Institute, Copenhagen, from pools of normal serum obtained from clotted blood taken by cardiac puncture. Fractional precipitation of the serum with barium acetate yielded gamma globulin which was demonstrated by paper electrophoresis to be essentially free of other protein components except traces of beta globulin.

The concentration of globulin was adjusted to approximately 1.0 gm. per cent. with physiological saline. This was given intravenously to 1.5 kgm. albino rabbits over a series of 12 injections. Following a rest period of 11 days, the animals were sacrificed and jugular blood was collected and pooled. The serum was tested for content of anti-guinea-pig gamma globulin by precipitin tests with purified guinea-pig gamma globulin. Using 0.1 ml. of undiluted antiserum, overlaid with 0.1 ml. quantities of 10-fold dilutions of the 1 per cent. globulin in saline, titres reached 1×10^6.

Preparation of the fluorescent rabbit anti-guinea-pig gamma globulin conjugate.

Antisera in these experiments were labelled with rhodamine-lissamine B200 (Imperial Chemical Industries, Ltd.) after the method of Chadwick et al., (1958). The rhodamine-conjugated rabbit antiserum was then treated with activated charcoal for 30 minutes. The ~~conjugated gamma globulin~~ preparation was thereafter

precipitated at 4° C. in half-saturated ammonium sulphate, centrifuged and re-dissolved in saline in one half the original volume of the serum utilised in the preparation. Dialysis was carried out at 4° C. overnight against physiological saline.

Acetone-dried bovine embryo kidney powder, prepared according to the method of Coons et al. (1955), in the amount of 0.1 gm. for each ml. of original serum, was then stirred into the ~~rhodamine-gamma-globulin~~ conjugate. After one hour the mixture was centrifuged. The supernatant was then re-stirred with one half the amount of kidney powder first used, for a period of one hour. The mixture was again centrifuged and the supernatant harvested. The absorbed rhodamine-labelled rabbit anti-guinea-pig gamma globulin was then titrated by precipitin tests against normal guinea-pig gamma globulin, as above. A titre of 10^5 was now obtained. The conjugate was stored at -20° C. until used.

Staining.

Fixation of coverslip preparations was carried out in acetone according to the method of Coons and Kaplan (1950). Uninfected preparations were removed from the tubes 12 hours after infection had been initiated in other tubes and treated similarly. The fixed preparations were stored at -20° C. until all the coverslips from the experiment had been fixed, so that staining could be carried out on all preparations simultaneously.

Fluorescent staining was carried out according to the indirect method of Weller and Coons (1954). Each preparation was overlaid

with appropriate antiserum, diluted 1 : 10 in phosphate buffered saline, pH 7.0. Guinea-pig antisera to the whole virus and to the purified S and V antigens, were utilised in separate preparations of each inoculum and time interval specified, and of uninoculated "12 hour controls". Uninoculated controls and inoculated preparations fixed 12 hours after infection were also overlaid with normal guinea-pig serum, to serve as additional controls. After 30 minutes of contact with the antisera the preparations were washed free of excess antibody, and then overlaid for 30 minutes with rhodamine-labelled rabbit anti-guinea-pig gamma globulin conjugate. Excess conjugate was washed free from the preparations with buffered saline, and the coverslips were then mounted on glass slides with a drop of buffered glycerine (9 vol. glycerine with 1 vol. buffered saline). They were observed for specific orange-red fluorescence under the ultra-violet-near-blue range, generated by an Osram HBO 200 mercury vapour lamp with filters. Control preparations showed no specific fluorescence.

Supersonic vibration. This was carried out in a Siemens Sonostat Standard. The apparatus was the same as that used by Mackeprang (1957). At the intensity employed, the energy output through the bottom of the cylindrical aluminium sample container has been calculated to be 0.56 watt/cm^2. at 0.8 mHz. Preliminary experiments showed that exposure of the virus to this intensity for 15 minutes had no influence on its stability (Lief and Henle, 1956a). Similar treatment of bovine embryo kidney cells caused complete disintegration.

Inactivation methods. Before inactivation, infected allantoic fluids were usually concentrated by adsorption on to chicken red cells and the virus was re-suspended in buffered saline containing 0.01 per cent. of a 10 per cent. solution of calcium chloride. Ultraviolet inactivation of these fluids was carried out as described by Henle and Henle (1947). Heat inactivation is described in the text.

Receptor destroying enzyme (RDE) was obtained from Behringwerke, Marburg, Germany.

EXPERIMENTAL

AND

RESULTS

PART I

NEWCASTLE DISEASE VIRUS IN HELA CELLS

The observation originally made by Tyrrell (1955), that Newcastle disease virus multiplies in HeLa cell monolayers, has been confirmed in this study. Experiments have shown that definite changes in the appearance of the cells follow the inoculation of cultures with the virus and both haemagglutinating and infective virus increase significantly when small inocula are introduced in the monolayers.

The cytopathic effect of the virus

In all the experiments concerning the NDV-HeLa cell system a regular sequence of changes was apparent, alike with neat or dilute allantoic fluid or culture fluid. The length of time for the effect to become visible was inversely proportional to the size of the inoculum but, even with small amounts of virus, cellular changes could be observed by 72 hours.

Experiment 1.

To examine the cytopathic effect of three strains of NDV.

HeLa cell cultures were inoculated with varying dilutions of three strains of NDV, i.e. Herts, Cal and B.1, as shown in Table 1 (Appendix). The tubes were incubated and were examined daily for cytopathic changes.

The cytopathic sequence of the Herts strain fell readily into the three stages described below. These are illustrated by Figs. 6, 7 and 8 which are photomicrographs of unstained infected cells; Figs. 4 and 5 show the appearance of uninfected

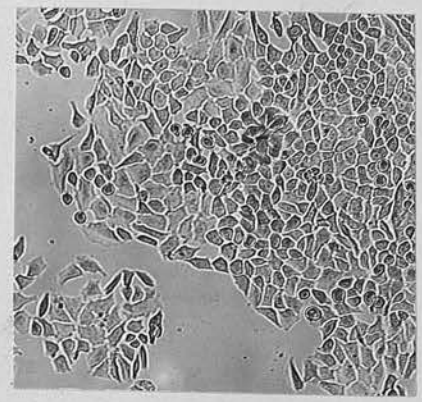

Figure 4.

Figure 5.

Normal unstained HeLa cells x 70.

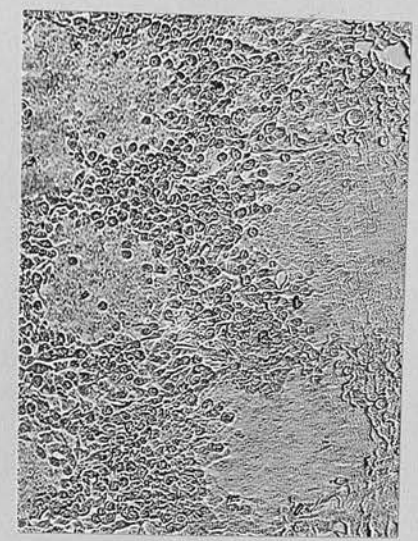

Figure 6.

24 hrs. after infection showing "microplaques".

Figure 7.

48 hrs. after infection showing "stellate effect".

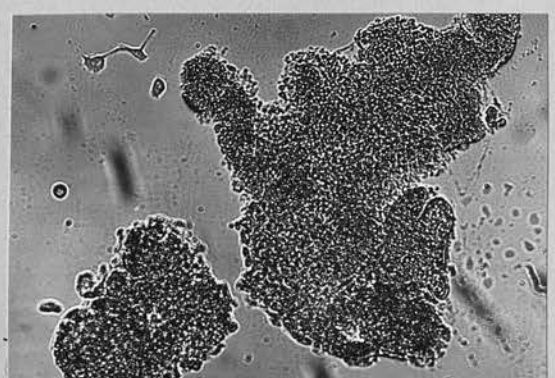

Figure 8.

72 hrs. after infection showing complete degeneration.

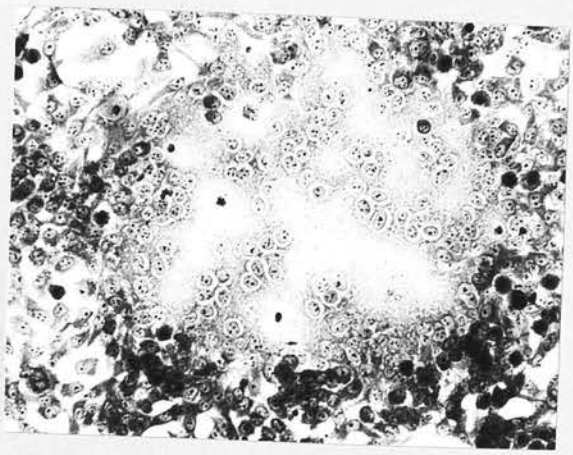

Figure 9.

HeLa cells 24 hrs. after infection with NDV showing the syncytial nature of a "microplaque". Giemsa's stain. x 140.

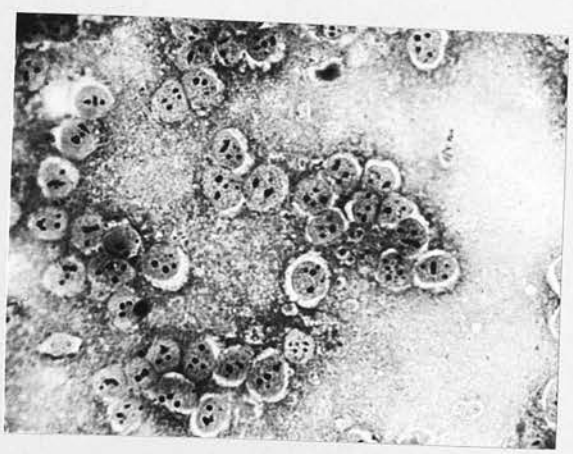

Figure 10.

Same as Figure 9.

x 360.

HeLa cell controls.

Stage 1. Many cells appeared normal but throughout the monolayer could be distinguished well-defined homogeneous areas of approximately 1 mm. in diameter (Fig. 6). These have been termed "microplaques".

Stage 2. The "stellate" effect produced at this stage apparently resulted from the shrinkage of the "microplaques". The continuity of the cell sheet was broken to release the ensuing tension (Fig. 7).

Stage 3. At this stage the "stellate" forms had contracted further to form clumps of debris, many of which had become detached from the glass.(Fig. 8). A few surviving cells could occasionally be seen.

When stained preparations were made of Stage 1 and examined under low and high power, the "microplaques" were seen to be of a syncytial nature; the cell boundaries could not be distinguished and the nuclei were aggregated in clumps (Figs. 9 and 10).

The virulent Cal strain produced cytopathic changes similar to those obtained with the Herts strain, whereas the avirulent B1 strain produced a slower and slightly different effect. Small areas of cytolysis occurred with clumping of the cells but definite "microplaques" were not apparent. The Herts strain was used in all subsequent experiments.

Time-lapse studies.

Further confirmation of the nature and sequence of the

cellular changes induced by the Herts strain of NDV in HeLa cells was obtained by time lapse cinematographic studies using the interference microscope.

Experiment 2 (see enclosed film).

To examine the cytopathic effect of the Herts strain of NDV by means of time-lapse cinematography.

HeLa cells in a slide culture chamber (Constable and Moffat, 1958) were infected with NDV allantoic fluid diluted 1 : 100 in Hanks' solution. Filming was then started and was continued over a 24-hour period. The film which was exposed at 30 second intervals, shows the gradual disappearance of the cell boundaries (Stage 1), the contraction of the resulting syncytium, giving the "stellate" appearance (Stage 2), and finally, the clumping of the cellular debris and its detachment from the glass (Stage 3).

The addition of homologous antiserum to the virus suspension before inoculation neutralised the cytopathic changes described above (see Experiment 12).

Correlation of cytopathic effect (CPE) with virus production.

The finding that the length of time for the cytopathic effect to become visible was inversely proportional to the amount of virus present in the inoculum indicated that HeLa cells might provide a suitable system for the titration of NDV. It was essential, however, to determine whether or not cellular damage provided an accurate indicator of the presence of virus.

Experiment 3.

To determine the correlation between development of CPE and haemagglutinins in HeLa cells infected with NDV.

HeLa cell cultures containing approximately 10^5 cells were inoculated with 0.1 ml. of NDV allantoic fluid in serial tenfold dilutions; 3 tubes were used for each dilution. The cells were examined daily for CPE and the medium was examined by a spot test for the presence of haemagglutinins.

The results are shown in Table 2 (Appendix). It can be seen that the tubes showing early cytopathic changes were negative in the haemagglutination test, although it is possible that these early haemagglutinins may have been diluted out. By the time the second stage of degeneration was reached, haemagglutinins were measurable, and the production of CPE would thus appear to be a reliable indicator of the growth of the virus. It seemed of interest that, with the more concentrated inocula (10^{-1}, 10^{-2}, 10^{-3}), haemagglutinins were apparently produced over a relatively short duration and after 4 days the cellular degeneration did not increase but seemed to come to a halt. Many apparently normal cells remained in the cultures. With increasing dilution haemagglutinins were measurable over a progressively longer period, and cellular degeneration appeared to be greater with the production of much debris.

These results suggested the occurrence of an interference phenomenon but there was no reason why this should hinder the

use of the CPE of NDV in HeLa cells as a means of titrating the virus. The results obtained by this method were reproducible within one log. The next experiment shows the correlation between the titres obtained in HeLa cells and in chick embryos.

Experiment 4.

The correlation between the titres obtained in HeLa cells and in chick embryos.

Parallel titrations of 0.1 ml. amounts of NDV allantoic fluid were carried out in HeLa cell cultures and in 9-day chick embryos. The titre per ml. obtained in HeLa cells was 8.0 TCD_{50} and in eggs was 9.4 EID_{50}. In subsequent experiments the EID_{50} was usually found to be higher than the TCD_{50} by approximately 1.5.

The multiplication of NDV in HeLa cells.

Having ascertained that HeLa cells could be employed for titrating NDV, thereby saving considerable labour and material, growth curve experiments were carried out. As the presence of an interference phenomenon had previously been indicated in this system, varying multiplicities of infection were included.

Experiment 5.

The effect of dilution on the infectivity, haemagglutinating activity and CPE of NDV in HeLa cells.

NDV allantoic fluid, neat and diluted 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}, was inoculated into HeLa cell cultures in 0.1 ml. amounts - 20 tubes per dilution. At daily intervals for 5 days:

a) the cells were examined for CPE;

b) the fluid was removed from 3 tubes, tested for HA and stored at -35° C.; this was the fluid phase;

c) after removal of the fluids, 1 ml. versene was added to the tubes and the resulting suspensions were pooled:

   i) cell counts were carried out;

   ii) the remaining cell suspension was centrifuged, the supernatant discarded, 1 ml. Hanks' solution was added to the deposit and the resulting suspension was stored at -35° C.; this was the cellular phase.

The fluid and cellular phase were then titrated as follows: Fluid phase. After thawing, the suspensions were diluted and titrated for infectivity in HeLa cells.

Cellular phase. After thawing, the suspensions were transferred to Pyrex test-tubes, after which they were frozen and thawed five times, using a mixture of solid CO_2 and methylated spirits. The resulting cell lysates were diluted in Hanks' solution and titrated in HeLa cells. HA tests were also carried out but the results of these tests cannot be relied on owing to the effects of freezing and thawing on the haemagglutinating property.

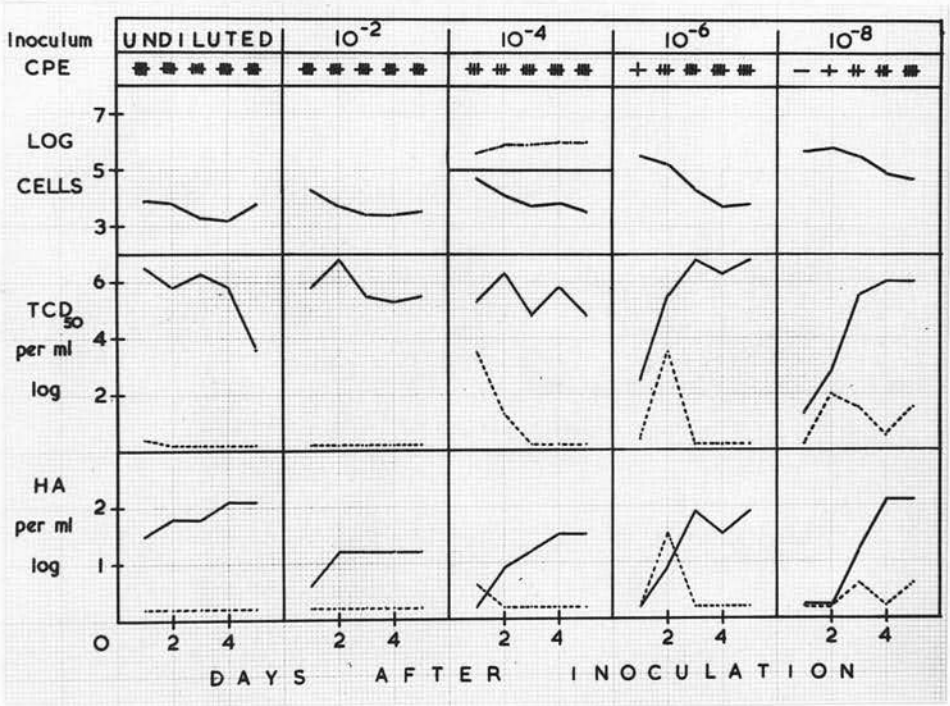

Figure 11.

The development of infectivity and haemagglutinating activity in the cells and in the culture medium after infection of HeLa cells with varying dilutions of NDV. The effect of the size of inoculum on the number of cells surviving is also shown.

_____ fluid phase

\- - - - - cellular phase

-.-.-.-.- uninfected controls.

The results of this experiment are shown in Table 3 (Appendix) and are graphically represented in Fig. 11. The cell counts show that the number of cells remaining in the cultures at a given time was inversely proportional to the amount of virus in the inoculum; this suggests a toxic effect. With the higher dilutions, (10^{-6} and 10^{-8}), as much, if not more, virus was produced than in the lower dilutions, with less damage to the cells. In the fluid phase it was impossible to detect any increase in infective virus with the large inocula but production of haemagglutinating virus and gross cellular damage were apparent; with smaller inocula a significant increase in infective virus and haemagglutinins was observed. The situation occurring with the cellular phase was not so easily interpreted but it would appear that, with large inocula, the cells became more quickly damaged so that there were few cells left on the wall of the tube in which virus could be measured. A rise in infective and haemagglutinating virus could be detected in the higher dilutions but this did not continue. The ratios obtained in the cellular phase were very low due, presumably, to the action of freezing on the virus and perhaps to the release of intracellular non-infectious haemagglutinins.

On comparing the results obtained with large and small inocula, it was found that with a large dose there was a detectable rise in haemagglutinin with no measurable rise in infective virus; with a small inoculum infective virus and haemagglutinins appeared concomitantly. These results indicated that incomplete

virus might have been produced following a heavy inoculum and thus suggested the presence of a von Magnus phenomenon.

The possibility that a von Magnus phenomenon might occur in the NDV-HeLa cell system was investigated in the following experiment.

Attempts to induce incomplete virus production.

Experiment 6.

The development of infectivity, haemagglutinating activity and CPE in HeLa cells infected with large and small inocula of NDV.

Two groups of 45 HeLa cell cultures were inoculated with different doses of NDV; one containing 6.5 TCD_{50} and the other with 1.25 TCD_{50}. At timed intervals 3 tubes were randomly selected from each group and after the cells had been examined microscopically for CPE, the fluids were harvested and tested for infectivity in HeLa cells and for haemagglutinating activity.

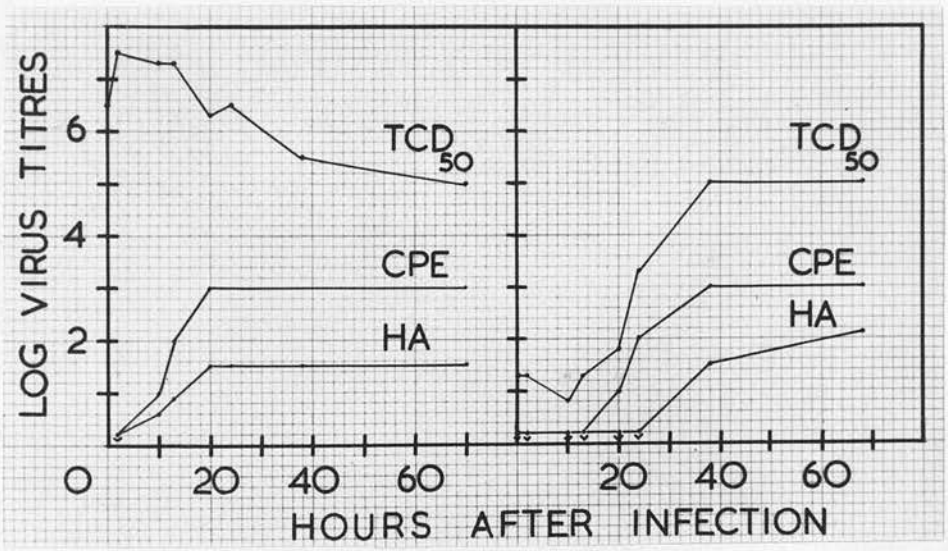

Figure 12.

The development of infectivity, haemagglutinating activity and CPE in HeLa cells infected with large and small inocula of NDV.

From the growth curves shown in Fig. 12 it may be seen that an increase in the amount of infective virus was only demonstrable when small doses were used. When massive doses were

given there was no significant increase in infective virus although haemagglutinins increased appreciably. In both cases, however, the completion of the cytopathic sequence was associated with the release of haemagglutinins although the time interval before the first appearance of cellular changes was inversely related to the size of the inoculum.

At first sight these results suggest a von Magnus effect, but on examining them further such an interpretation is not fully justified. Owing to the size of the inoculum and the corresponding amount of residual virus present in the medium, any increase in infective virus is difficult to measure; heat inactivation must also be taken into account. A feature which was evident in this and subsequent experiments was the inability to detect haemagglutinins early in the growth cycle in spite of the infectivity titre being over 7 TCD_{50}. It is possible that some inhibitor, removed later by the change in pH, was responsible.

In a further attempt to secure evidence to support or refute the existence of a von Magnus effect in this system, serial passage experiments were carried out.

Experiment 7.

Serial passage of NDV in HeLa cells using large and small inocula.

NDV allantoic fluid was diluted in Hanks' solution 10^{-1} and 10^{-6} and these dilutions were passed serially in HeLa cell cultures. After each passage the fluids were harvested and titrated and

passed further, either diluted or undiluted, as shown in the scheme in Table 4.

TABLE 4. Serial passage of NDV in HeLa cells using large and small inocula.

DATE	PASS. NO.	DILUTED 10^{-6}			DILUTED 10^{-1}		
		DILUTION	HARVESTED	TCD_{50}	DILUTION	HARVESTED	TCD_{50}
13.3.58.	1	-6	3 days	4.3	-1	3 days	2.5
20.3.58.	2	-4	5 "	2.8	-1	5 "	2.3
29.3.58.	3	-2	4 "	3.5	-1	4 "	3.5
2.4.58.	4	-2	3 "	4.0	-1	3 "	3.5

The infectivity titres throughout 4 passages are shown in Fig. 13.

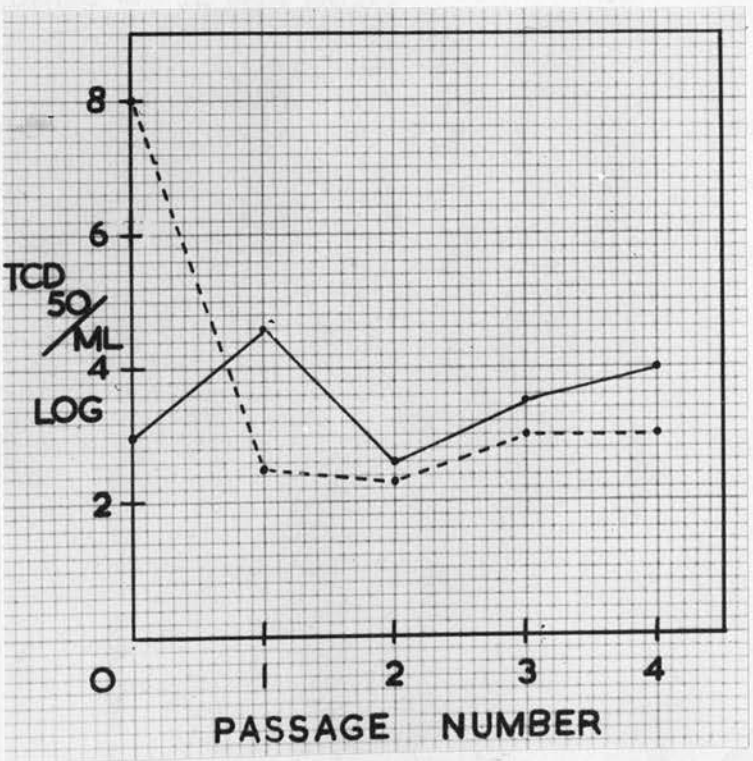

Figure 13.
The effect of serial passage in HeLa cells on the infectivity titre of NDV.

- - - - - large inoculum

——— small inoculum

During the first passage the large inoculum gave rise to no increase in infective virus whereas a rise could be demonstrated with the more dilute inoculum. In subsequent passages, however, no significant differences could be demonstrated between these two series suggesting that no alteration had occurred in the quality of the virus during the first passage. Satisfactory evidence for the presence of a von Magnus phenomenon was not obtained from these results.

At this stage it would appear essential to obtain some idea of the rate at which NDV in different concentrations loses its power to produce cytopathic effect.

Experiment 8.

Heat inactivation of NDV as measured by the loss of ability to induce CPE in HeLa cells.

NDV allantoic fluid was used in dilutions 10^{-1} and 10^{-6}. Test-tubes containing 0.9 ml. medium received 0.1 ml. amounts of these virus suspensions and were then incubated at 37° C. At timed intervals the fluids were tested for CPE in duplicate HeLa cell cultures and these cultures were examined over 72 hours for cellular changes.

TABLE 5. The effect of incubation at 37° C. on the cytopathic effect of NDV in HeLa cells.

HOURS' INCUBATION AT 37° C.	PRODUCTION OF CPE	
	10^{-6}	10^{-1}
18	+	+
42	-	+
68	-	+
92	-	-

It may be seen from Table 5 that the ability to induce cytopathic changes was lost by the 10^{-6} dilution between 18 and 42 hours and by the 10^{-1} dilution between 68 and 92 hours.

Survival of HeLa cells after treatment with NDV.

Previous observations made in this study, that more cells survived infection with a large dose of virus than with a small dose of virus, suggested the necessity for some further experiments on the survival of cells after infection with NDV.

Experiment 9.

The survival of HeLa cells after inoculation with varying doses of NDV.

NDV allantoic fluid in dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} was added to HeLa cell cultures in 0.1 ml. amounts from which the nutrient medium had been removed. The tubes were incubated in the roller drum at 37° C. for 30 minutes after which time 0.9 ml. nutrient medium was added to each tube and they were re-incubated at 37° C. The cells were examined daily for CPE and after 3 days, by which time all tubes showed complete degeneration and positive haemagglutination, the culture fluids were harvested and titrated for infectivity in the usual way. After removal of the virus from the cultures, there were still many surviving cells on the wall of the tube. The tubes were then washed with Hanks' solution and 1 ml. of fresh nutrient medium was added to each tube. The cultures were finally incubated in the roller drum at 37° C. and were examined daily for growth of cells.

The results are shown in Table 6 (Appendix). From these results it would appear that, although the extent of CPE was greatest with a large inoculum of virus, the effect was halted in some way and as many, if not more, cells were capable of

surviving the infection than in the case of a more dilute inoculum. This finding may be related to the fact that less infective virus was produced following a large inoculum and again indicated the presence of an interference phenomenon which was duely perhaps, to an overloading of the cells with virus.

A further experiment to confirm these findings is described below.

Experiment 10.

The number of HeLa cells surviving after infection with varying doses of NDV.

NDV allantoic fluid, in dilutions 10^{-2} and 10^{-4}, was added to two groups of HeLa cell cultures; uninfected controls were also included. The tubes were incubated in the roller drum at 37° C. and at intervals were examined for CPE and haemagglutinating activity. At the same time cell counts were carried out after adding 1 ml. versene to two tubes from each group.

TABLE 7. The number of HeLa cells surviving after infection with NDV.

DILUTION	DAYS AFTER INOCULATION										
	CPE				HA				Cell Count x 10^4		
	1	2	3	4	1	2	3	4	1	2	
10^{-2}	+++	+++	+++	+++	+++	+++	+	+	3.7	1.8	1.0
10^{-4}	+	+++	+++	+++	-	+++	+	+	60	2.3	0.6
Control	-	-	-	-	-	-	-	-	90	100	100

The results of this experiment, which are shown in Table 7, confirm the previous findings by showing that more cells remained

four days after infection with a large inoculum than after a smaller inoculum.

A further experiment in this series was planned to ascertain whether HeLa cells would grow out after previous treatment with NDV, and if so, whether the dilution had any effect.

Experiment 11.

The growth of HeLa cells after previous treatment with NDV.

5 ml. amounts of a HeLa cell suspension containing approximately 10^5 cells per ml. were placed in each of three flasks to which were added:

Flask no. 1 : 1 ml. Hanks' solution

" " 2 : 1 ml. NDV 10^{-4}

" " 3 : 1 ml. NDV 10^{-1}

The contents of the flasks were mixed and were kept at 4° C. for 1 hour. After this, HA tests were carried out and 4 test-tubes were inoculated with 0.5 ml. of each suspension and incubated in a stationary position. The remainder of the fluid was left at room temperature (20° C.) for $1\frac{1}{2}$ hours; at the end of this time HA tests were again carried out and 0.5 ml. of each suspension was inoculated into tubes as before. Microscopical examinations, haemagglutination tests and cell counts were carried out at intervals on the newly inoculated cultures.

The results of this experiment are shown in Table 8(Appendix) and the effect of the virus on the cells may be clearly seen. In contrast to the previous experiments, a greater number of cells survived after treatment with a small inoculum; this

confirmed the idea that the preliminary cellular destruction might be due to a toxic effect. Haemagglutinins were produced in all cases even after such a slight growth of the cells; HA production was greater, however, in the cells treated with a higher dilution of virus. This was due, presumably, to the fact that there were more cells available for virus production.

An attempt to measure the amount of NDV adsorbed to HeLa cells failed, due to the insensitivity of the titration techniques employed.

Neutralisation experiments.

It has been mentioned previously that the CPE of NDV in HeLa cells may be neutralised by homologous antisera and a typical neutralisation test is described below.

Experiment 12.

Neutralisation of the CPE of NDV in HeLa cells by homologous antiserum.

NDV immune serum was diluted 1 : 10, 1 : 100, 1 : 250, 1 : 500 and 1 : 1000. These dilutions were mixed with equal quantities of NDV allantoic fluid diluted 1 : 100 and left at room temperature for one hour. The mixtures were then added to HeLa cells in 0.1 ml. amounts. The cells were examined daily for CPE and the fluids were tested for HA.

The results are presented in Table 9 (Appendix). It may be seen that the neutralising titre of the antiserum for 1 : 100 virus was between 1 : 10 and 1 : 100.

Further neutralisation tests of this type were planned to determine whether or not the antiserum would still be capable of reducing the cytopathic effect of the virus if added to the cells after infection.

Experiment 13.

Neutralisation of the CPE of NDV in HeLa cells by antiserum added after infection with the virus.

Two groups of HeLa cell cultures, one inoculated with a 10^{-6} dilution of NDV allantoic fluid and the other with a 10^{-9} dilution, were incubated for 2 hours after infection. After this

time the medium was removed and the cells were washed with balanced Hanks' solution. The two groups of cultures were then divided into 5 groups as shown in Table 10 (Appendix); each of 4 of these groups received 0.1 ml. amounts of antiserum in dilutions 10^{-2}, 10^{-3}, 10^{-4} or 10^{-5}. The 5th group received no antiserum and acted as virus controls. All tubes received 1 ml. fresh nutrient medium and the cells were incubated for 3 days. During this period the cells were examined for CPE.

The results presented in Table 10 show a striking reduction in CPE in the presence of antiserum; the reduction was proportional to the amount of antiserum present, being most marked with the 10^{-2} dilution of antiserum, whereas with the 10^{-5} dilution the effect was not detectable. Confirmatory experiments using higher concentrations of virus and antiserum were carried out and are described below.

Experiment 14.

Neutralisation of the CPE of NDV in HeLa cells by antiserum added after infection with the virus.

Two groups of HeLa cell cultures, one inoculated with a 10^{-3} dilution of NDV allantoic fluid and the other with a 10^{-7} dilution, were incubated for 30 minutes after infection. The fluid was then removed, the cells washed with Hanks' solution and 1 ml. new medium added. The two groups of cultures were then divided into 3 groups as shown in Table 11 (Appendix); each of two of these groups then received either 10^{-1} or 10^{-2} antiserum in 0.1 ml. amounts; the third group acted as virus controls. The tubes were incubated at 37° C. and the cells were examined

at intervals for CPE. No HA tests were performed on the fluids.

The cellular changes observed in the above experiment over a period of 8 days are shown in Table 11. It was again demonstrated that the addition of antiserum to the cultures reduced the amount of CPE produced in a given time and that the extent of reduction was dependent on the amount of antibody present.

The effect of sodium metaperiodate on the growth of NDV in HeLa cells.

Potassium metaperiodate has been found to modify cell receptors, thereby preventing infection by influenza virus (Fazekas de St. Groth and Graham, 1949). As it was thought feasible that similar treatment might prevent infection of HeLa cells by NDV, some experiments on these lines were carried out.

Experiment 15.

The CPE and growth of NDV in periodate-treated HeLa cells.

Twelve out of a group of 24 HeLa cell cultures were treated with sodium metaperiodate as follows: the medium was removed and to each tube was added 1 ml. sodium metaperiodate (0.01 M). The tubes were incubated in the roller drum for 15 minutes, after which the excess periodate was neutralised by the addition of 1 ml. glycerol saline. The fluid was removed and was replaced by 0.9 ml. nutrient medium. Treated and untreated cells were then incubated with virus as shown in Table 12 (Appendix) and incubated. The cultures were examined daily for CPE and after 3 days' incubation the fluids were harvested, pooled and centrifuged; the supernatants were titrated in HeLa cells and tested for HA.

The results presented in Table 12 demonstrate that the treatment of HeLa cells with sodium metaperiodate previous to infection with NDV prevented multiplication of the virus and the production of the characteristic cytopathic sequence. On the other hand, although the treated cells retained their normal

shape even in the presence of virus, they tended to slip off the glass and had a somewhat shrunken appearance. An attempt was therefore made to inhibit the multiplication of NDV employing a less concentrated solution of $NaIO_4$.

Experiment 16.

The CPE and growth of NDV in periodate-treated cells.

The method used was similar to that used in the previous experiment except that 0.5 ml. amounts of either 0.01, 0.001 or 0.0001 M $NaIO_4$ were added to the tubes, which were incubated for 10 minutes. Excess periodate was neutralised by the addition of 0.5 ml. glycerol saline (0.01 M).

The results of this experiment are shown in Table 13 (Appendix). The higher dilutions of $NaIO_4$, i.e. 0.001 and 0.0001 M, were found to have no inhibitory effect on the CPE of NDV, whereas the 0.01 M solution, although preventing the CPE as before, still induced in the cells a slightly shrunken appearance. The following experiment was therefore carried out to explore the possibility of using a dilution between 0.01 M and 0.001 M.

Experiment 17.

The CPE and growth of NDV in periodate treated HeLa cells.

The experiment was carried out as Experiment 16, except that dilutions 1 : 2, 1 : 4, 1 : 8 and 1 : 10 of 0.01 M $NaIO_4$ were employed.

The results are shown in Table 14 (Appendix). The lowest dilution of 0.01 M sodium periodate which had some inhibitory effect on the CPE and on the production of haemagglutinins was

1 : 4. With this dilution, however, the control cells still had a shrunken appearance which indicated that the surface of the cells had been extensively altered in some way, thereby preventing the entry of the virus. Beyond the 1 : 4 dilution no evidence for suppression of virus manifestation was obtained.

Liu et al. (1956) have reported that, with influenza virus, the particle itself is affected by potassium periodate. It was necessary at this stage, therefore, to find out if a similar situation obtains with NDV.

Experiment 18.

The effect of sodium metaperiodate on the NDV particle.

NDV allantoic fluid was diluted 10^{-1} and 10^{-6}. These dilutions were treated with 0.01 M $NaIO_4$ and after 10 minutes glycerol saline (0.01 M) was added. The mixtures were then tested for HA and 0.1 ml. amounts of each suspension were added to HeLa cells to test for CPE. Saline-treated controls, as shown in Table 15, were also included.

TABLE 15. The effect of sodium metaperiodate on the properties of NDV.

MIXTURE			HA after mixing	CPE after 3 days
SALINE	$NaIO_4$	VIRUS		
0.5 ml.		0.9 ml. 10^{-1}	2.1	+++
0.5 ml.		0.9 ml. 10^{-6}	-	+++
	0.5 ml.	0.9 ml. 10^{-1}	1.8	+++
	0.5 ml.	0.9 ml. 10^{-6}	-	-

It is apparent from the results shown in Table 15 that the virus particle had been affected by the periodate. In the presence of periodate the haemagglutinating activity of the 10^{-1} dilution was lowered while the CPE of the 10^{-6} virus had apparently been removed. The CPE of 10^{-1} virus was not altered. This suggests that the suppression of the CPE of Newcastle disease by sodium metaperiodate demonstrated in Experiments 15, 16 and 17 may have been partly due to the action of the chemical on the virus and partly due to its modification of the cell surface.

EXPERIMENTAL

AND

RESULTS

PART II

INFLUENZA A VIRUS IN MONKEY KIDNEY CELLS, BOVINE EMBRYO KIDNEY CELLS, AND HELA CELLS.

Monkey kidney tissue culture has been used extensively in recent years for the isolation and propagation of the influenza viruses (Mogabgab et al., 1955; Takemoto et al., 1955; Mogabgab et al., 1956; Green et al., 1957, a, b) and has been shown to support the replication of complete influenza virus. Calf or bovine embryo kidney cells also offer a suitable host cell system (Green et al., 1957; Haas and Wulff, 1957; Warren and Cutchins, 1957; Heath and Tyrrell, 1958). These two tissue culture systems have been employed in the present series of experiments to examine various aspects of the growth of influenza virus.

Normal growth of influenza virus in tissue culture.

Experiment 19.

To obtain a growth curve of the virus using a dilute inoculum.

Cultures of monkey kidney cells and bovine embryo kidney cells containing 1.8 ml. nutrient medium and 11-day chick embryos were inoculated with 0.2 ml. of a 10^{-4} dilution of standard PR8 virus and incubated at 35.5° C. At 6 hour intervals the culture fluids from the tissue cultures and the allantoic fluids from the eggs were harvested and tested for egg infectivity and haemagglutinating activity. The cells were also examined for cytopathic changes.

The growth curves of the virus in the three host systems are shown in Figure 14.

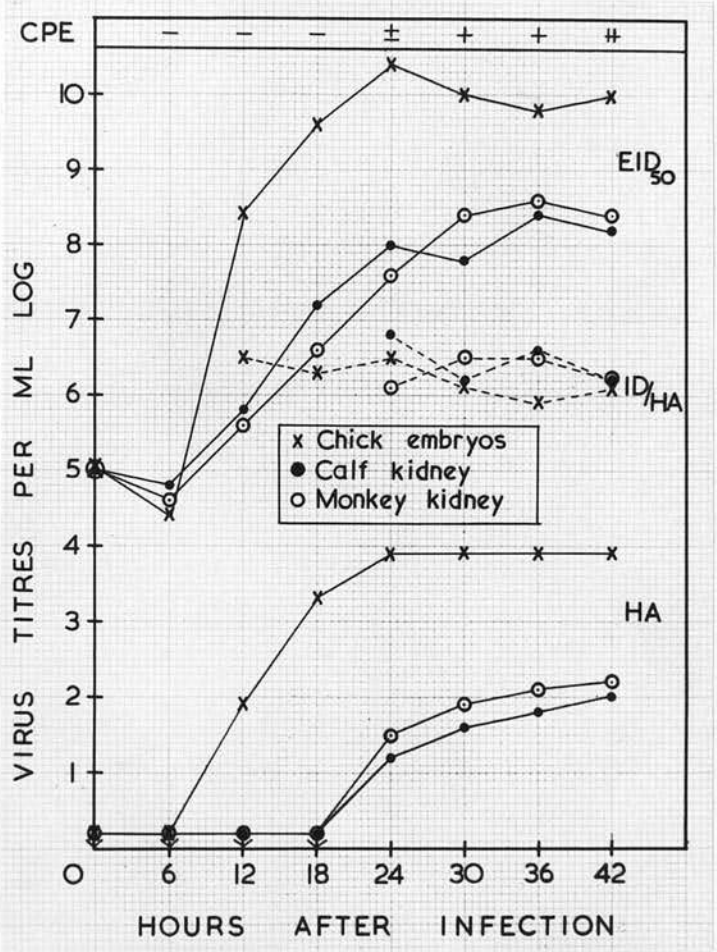

Figure 14.
The development of egg infectivity and haemagglutinating activity in fluids harvested from chick embryos, monkey kidney cultures and bovine embryo kidney cultures after inoculation with a 10^{-4} dilution of standard PR8 virus. The CPE observed in the cultures is also indicated.

It may be seen that the growth of the virus in monkey kidney cells and bovine embryo kidney cells was almost identical. Haemagglutinins were first detectable 24 hours after infection and at that stage cytopathic effects were apparent. The amount of infectious virus in the medium reached a peak at approximately 36 hours, by which time cytopathic changes were easily seen;

many of the cells were rounded and in clumps but complete destruction was never encountered after a dilute inoculum. In eggs, the virus grew more rapidly and reached a significantly higher titre than in tissue culture; haemagglutinins were first detectable at 12 hours and the amount of infectious virus reached a peak at 24 hours.

From these results it is apparent that, although quantitative differences occurred in the three host systems investigated, the quality of virus produced was comparable; in all cases the ratios of egg infectivity to haemagglutinating activity (ID/HA) were of the order of 10^6.

The significantly lower titre obtained in tissue culture suggested the possibility of adapting the virus to growth in monkey kidney cells. This has previously been reported for influenza A virus (Green et al., 1955; Heath and Tyrrell, 1958) and for influenza B virus (Green et al., 1957).

Experiment 20.

An attempt to adapt the virus to growth in tissue culture.

Monkey kidney cultures were inoculated with 0.5 ml. of a 10^{-4} dilution of allantoic fluid and the cells were washed after 2 hours. After 72 hours' incubation the fluids were harvested, titrated for infectivity in eggs and in tissue culture and for haemagglutinating activity. The pooled fluid was diluted 10^{-4} and added to more monkey kidney cultures in 0.5 ml. amounts; the virus was carried in this way through 21 passages.

The results which are presented in Table 16 (Appendix) show that, when the EID/HA ratio was compared with the TCD/HA ratio to give an EID/TCD ratio, no significant alteration throughout the serial passages could be detected. In this particular experiment there are no values for the egg infectivity titres for the first 5 passages but in previous experiments these passages had been shown to be similar. In all cases the EID/HA ratio was of the order of 10^6 and the TCD/HA ratio was of the order of 10^4. The EID/TCD ratio was consequently of the order of 10^2; this was the difference observed when the growth curves of the virus were examined in eggs and in tissue cultures and would thus appear to be a constant factor.

In these experiments there was no evidence of adaptation of the PR8 strain of influenza virus to growth in monkey kidney cells.

Attempts to produce "incomplete" virus.

The following experiments were carried out to investigate whether or not incomplete virus or non-infectious haemagglutinins (NIHA) could be produced in monkey kidney tissue culture.

In the first group of experiments an attempt was made to reproduce in monkey kidney cultures the results which had been obtained in eggs by undiluted serial passage of the virus (von Magnus, 1951). A typical experiment is described below:

Experiment 21.

Serial passage of undiluted tissue culture fluid.

Two groups of monkey kidney cultures were inoculated with 0.2 ml. of undiluted allantoic fluid which had an approximate titre of 10^{10} EID_{50} per ml., giving a multiplicity of infection of approximately 10^4 egg infectious doses per cell. In group A the cells were washed after 2 hours while in group B the cells were not washed. Tissue culture fluids were sampled $2\frac{1}{2}$ hours after inoculation and were tested for egg infectivity and haemagglutinating activity. At 24 hours the cells were examined for CPE, the fluids were harvested and titrated and 0.2 ml. amounts of these pooled fluids were inoculated into more MK tissue cultures; this was continued throughout 17 passages.

The results of such a serial passage are shown in Table 17 (Appendix) and it will be seen that, although there were variations in the ratios obtained, there was no definite indication of incomplete virus production. In group A where the cells were washed, the ID/HA ratios were almost identical to those obtained in the dilute passage (see Table 16), whereas in

in group B the ratios were more often of the order of 10^5; this difference may be explained by the presence of residual heat-inactivated virus. The variations in titre occurred simultaneously and as the two series were carried concomitantly it seems possible that thinner cultures were responsible for the variations between the passages; for example, in group A no HA was detected after the 8th passage, while in group B only a small amount of HA was produced, but on repeating this passage in another batch of monkey kidney cells haemagglutinins were produced in the washed cultures. In all passages, there was heavy destruction of the cells at 72 hours after inoculation; this was most complete, however, in the first passage when undiluted allantoic fluid was used as inoculum.

Neither in this experiment nor in many similar experiments was there any evidence to suggest that a significant amount of incomplete virus had been formed. Further possibilities of producing incomplete virus were therefore explored. They may be summarised as follows:

Experiment 22:

standard virus was concentrated twenty-fold by adsorption on red cells before serial passage;

Experiment 23:

the virus was passed undiluted, i.e. the medium was removed from the cells prior to inoculation and 2 ml. of the neat infective fluid was added to each tube. This was carried out at 37° C. and 35° C.;

Experiment 24:

age of cells and time of incubation were varied.

In all these experiments ID/HA ratios between 10^5 and 10^6 were obtained.

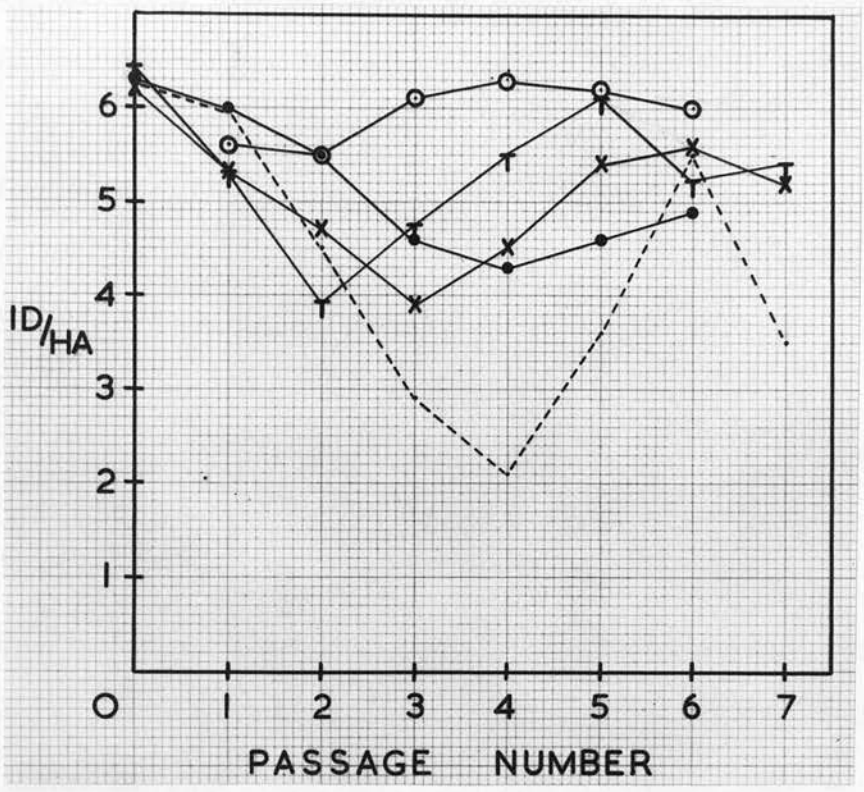

Figure 15.
The ID/HA ratio of PR8 influenza virus following undiluted serial passage in chick embryos and in monkey kidney (MK) tissue cultures.

	HOST SYSTEM	NO. OF CELLS	ORIGINAL INOCULUM	EID_{50}/CELL
O	MK bottles	10^6	5 ml. 1 : 10	10^3
●	" "	"	5 ml. UD	10^4
T	" tubes	10^5	0.5 ml. UD	10^4
X	" "	"	0.2 ml. UD	10^4
----	Chick embryos	10^7	0.5 ml. UD	10^3

However, during numerous serial passage experiments variations from the general pattern were encountered and are summarised in Fig. 15. The results obtained from a typical

serial passage in eggs are also included. Although a gradual decrease in ID/HA ratio was observed in some of the tissue culture passages, this was never so striking as that occurring in eggs and was not easily reproducible. It seemed at one stage as if the increase of the number of cells per ml. of medium by growing the cells in prescription bottles might induce detectable production of NIHA, but attempts to reproduce such findings gave variable results.

Experiment 25.

Passage of undiluted egg-passaged (UP) seeds in monkey kidney tissue culture.

In an effort to discover a more readily reproducible method of inducing incomplete virus, material from undiluted serial egg passages was inoculated into monkey kidney cultures.

TABLE 18. The effect of undiluted passage in eggs on the egg infectivity and haemagglutinating activity of PR8 influenza virus.

Egg passage	Inoculum ml.	Incubated hrs.	Titres per ml.log		
			EID_{50}	HA	ID/HA
Standard	0.2	42	10.2	4.2	6.0
UP 1	0.5	18	9.2	4.2	5.0
UP 2	0.5	18	8.4	4.0	4.4
UP 3	0.5	18	7.0	3.9	3.1
UP 4	0.5	18	6.2	3.0	3.2

The allantoic fluids from the standard egg passage and from the first four undiluted passages (UP 1, UP 2, UP 3, and UP 4) (see Table 18) were inoculated into monkey kidney cultures

in 0.2 ml. amounts. At intervals the cells were examined for CPE and the fluids were titrated for egg infectivity and haemagglutinating activity.

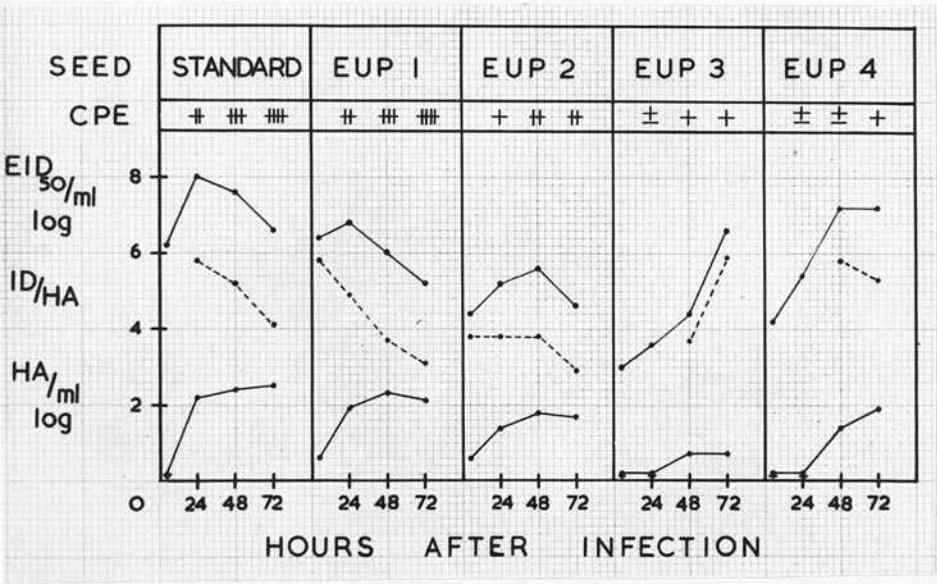

Figure 16.

The development of egg infectivity, haemagglutinating activity and CPE in monkey kidney cultures after inoculation with 0.2 ml. of PR8 influenza virus which had previously undergone standard and undiluted serial passage in eggs.

EUP = egg undiluted passage.

The growth curves are shown in Fig. 16. When standard virus was inoculated undiluted into the cultures, the EID_{50} and HA titres obtained were comparable to those reached when diluted standard virus was grown in monkey kidney cultures (see Fig. 14) although, on account of the size of the inoculum, they were reached earlier. The ID/HA ratio was of the order of 10^6 at 21 hours, after which time it decreased due to the action of heat on the infective particles. The CPE was quite marked at 21 hours and by 70 hours few surviving cells could be seen.

The growth curve obtained with UP 1 indicated that a certain proportion of incomplete virus had been produced, while that induced by UP 2 showed an ID/HA ratio of the order of 10^4, indicating a considerable production of NIHA. With UP 3 and UP 4 the proportion of complete virus produced again increased.

From this experiment it was evident that incomplete virus production could be induced in monkey kidney tissue culture by the use of seeds which had undergone undiluted serial passage in eggs.

The variation in CPE induced by these fluids should also be mentioned; with standard seed and UP 1 seed the cell sheet was almost completely destroyed; with UP 2, however, heavy destruction of the cells was never observed, while with UP 3 and UP 4 even fewer changes were evident, in spite of the fact that more infective virus was produced. This suggested a toxic effect associated with the presence of infectious virus in the inoculum.

When the growth curves shown in Figure 16 are compared with those in Figure 17, which were obtained from fluids having undergone previous serial passage in monkey kidney tissue cultures, it is seen that, although the production of NIHA was not so striking as that obtained when egg passaged fluids were employed, the general trend of the curves is similar.

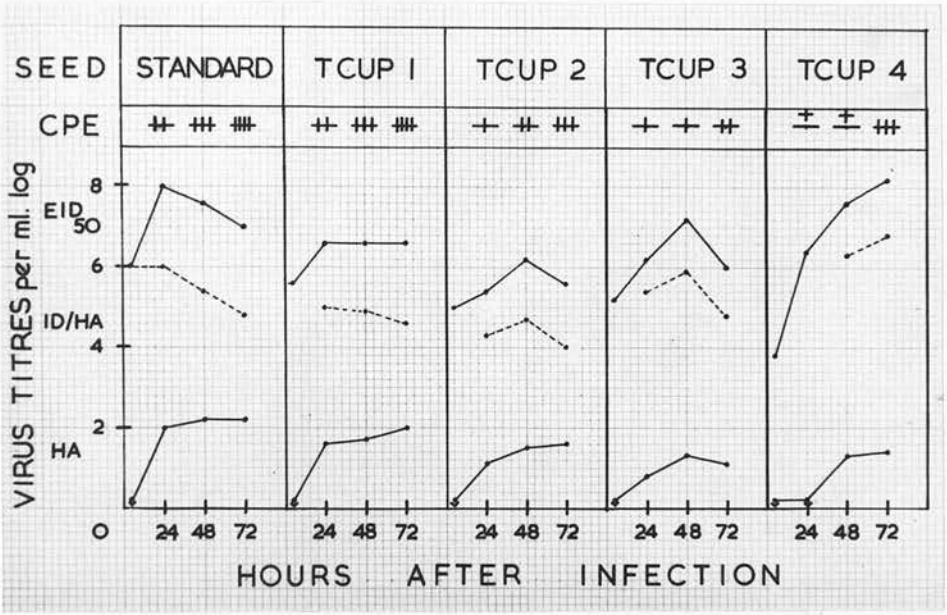

Figure 17.

The development of egg infectivity, haemagglutinating activity and CPE in monkey kidney cultures after inoculation with 0.2 ml. of PR8 influenza virus which had previously undergone undiluted serial passage in monkey kidney tissue cultures.

TCUP = tissue culture undiluted passage.

Further experiments were then carried out to study the growth of egg-passaged UP 2 (EUP 2) in monkey kidney cells, since in both eggs and tissue cultures it was this fluid which induced the highest proportion of NIHA in the succeeding passage.

Experiment 26.

Growth curves of EUP 2 in eggs, monkey kidney and bovine embryo kidney cells.

Undiluted UP 2 allantoic fluid in 0.2 ml. amounts was inoculated into monkey kidney and bovine embryo kidney cultures which contained 1.8 ml. nutrient medium, and into the allantoic cavity of 11-day chick embryos. At 6-hour intervals the cells

were examined for CPE and the fluids removed and titrated for egg infectivity and haemagglutinating activity.

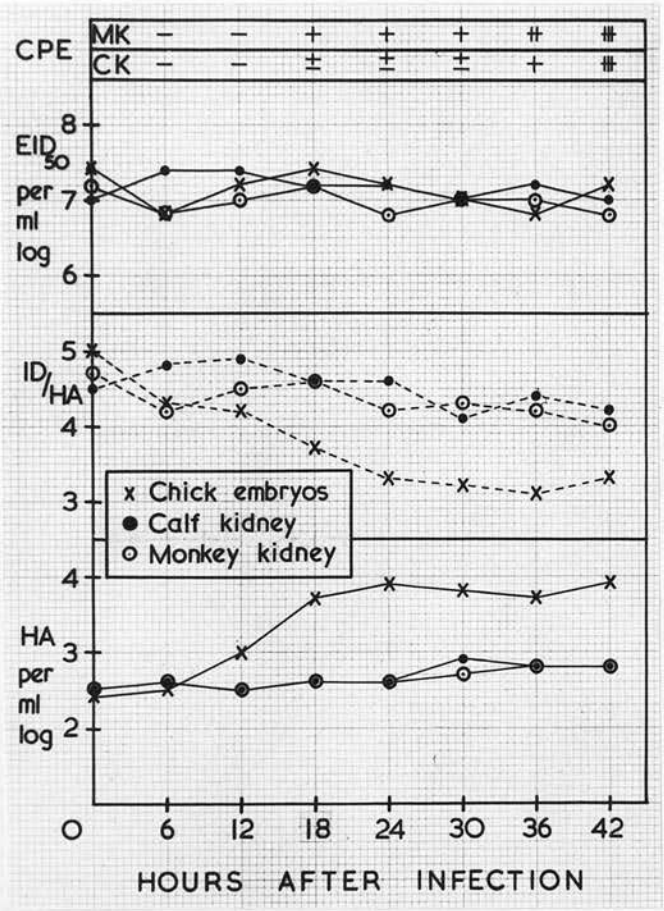

Figure 18.
The development of egg infectivity and haemagglutinating activity in fluids harvested from chick embryos, monkey kidney cultures and bovine embryo kidney cultures after inoculation with PR8 allantoic fluid which had undergone two undiluted passages in eggs (EUP 2). The CPE observed in the tissue cultures is also indicated.

The growth curves shown in Fig. 18 provide evidence of depressed growth of the virus, especially in the case of embryonated eggs, where a significantly greater amount of NIHA was produced.

Experiment 27.

Effect of dilution on the growth of EUP 2 in monkey kidney cells.

EUP 2 was inoculated undiluted and in dilutions 10^{-1} and 10^{-2} into monkey kidney cultures; the cells were washed after 2 hours. At intervals after infection of $2\frac{1}{2}$ hours, 23 hours, 47 hours, and 70 hours, the cells were examined for CPE and the fluids harvested and titrated for egg infectivity and haemagglutinating activity.

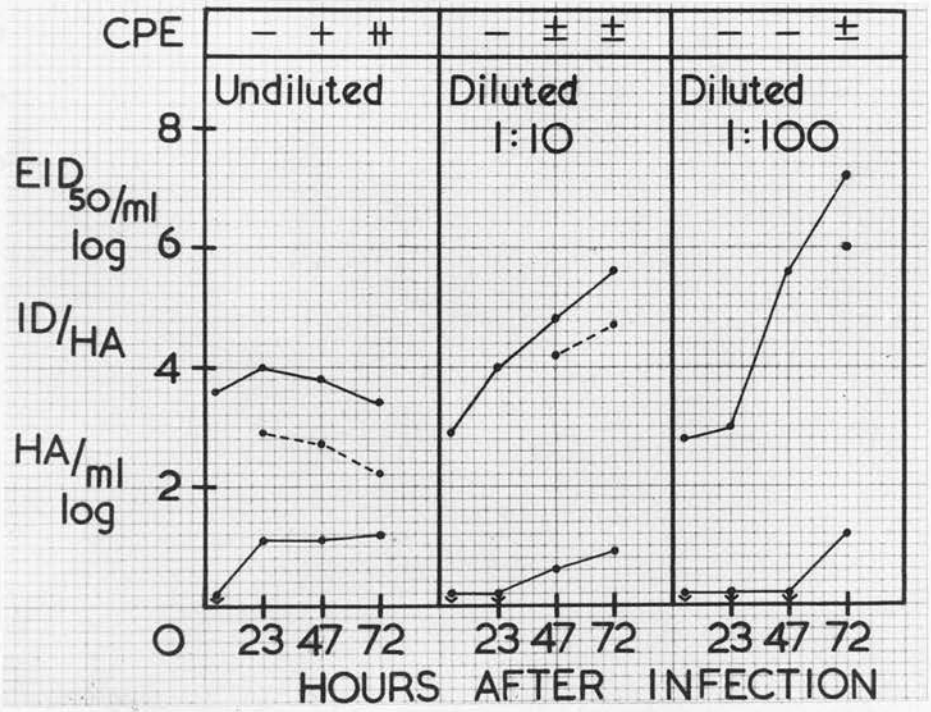

Figure 19.
The effect of dilution on the growth curve of EUP 2 in monkey kidney cultures.

The results are shown in Figure 19 and demonstrate that the effect responsible for depressed multiplication of EUP 2 may be diluted out. A 10^{-2} dilution of the seed resulted in the production of fully active virus.

Experiment 28.

Effect of purification of the EUP 2 seed on its growth in monkey kidney cultures.

In order to investigate whether or not the capacity to produce NIHA was directly related to the virus particle, the EUP 2 seed was partially purified by adsorption on to chicken red cells.

Following two adsorptions of EUP 2 on to chicken red cells, the resulting eluates and supernates were inoculated in 0.2 ml. amounts into monkey kidney cultures. Control cultures were inoculated with unadsorbed EUP 2.

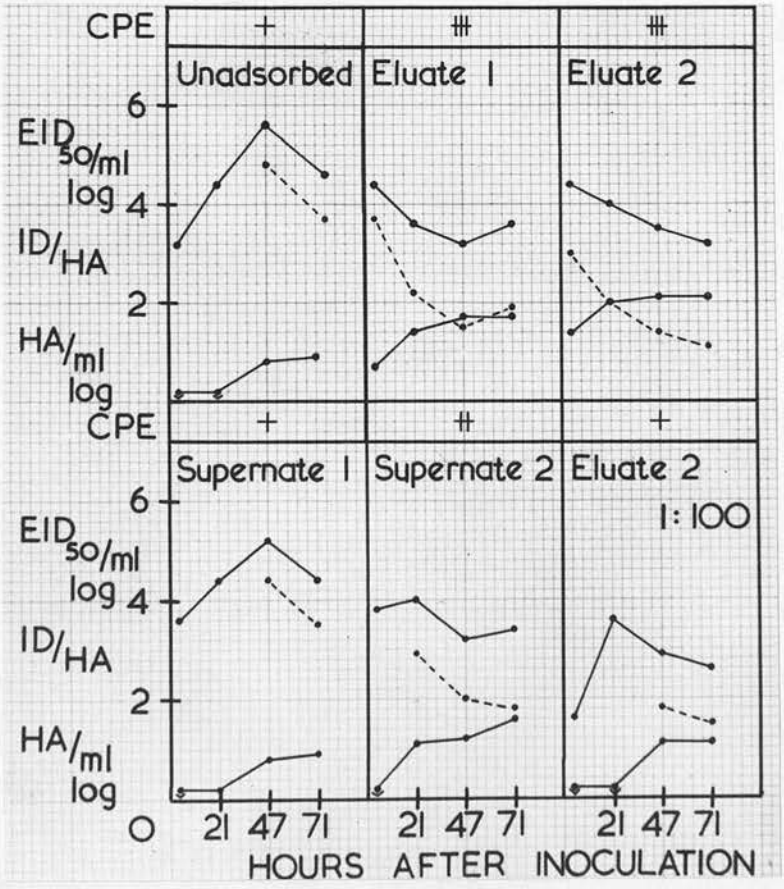

Figure 20.
The effect of purification by red cell adsorption on the growth of EUP 2 in monkey kidney cultures.

From the growth curves which are shown in Fig. 20 it may be seen that in all cases the production of infectious virus was depressed; there was considerable production of NIHA by the second eluate and even when this fluid was diluted 1 : 100 a significant amount of NIHA was produced (see Fig. 19). These results indicate that the phenomenon is directly related to the virus particle and are in agreement with previous findings (von Magnus, 1951 b). The heavy cellular destruction induced by the eluates in the absence of normal infectious virus production seems of interest.

Experiment 29.

The growth in monkey kidney culture of fluids partially inactivated by heat.

It has been shown by Paucker and Henle (1955) that standard seeds of influenza virus, after heating for several days at 37° C., resemble in many respects those obtained by undiluted serial passage in eggs (von Magnus, 1951). When undiluted passage (UP) and heated standard seeds were compared on the basis of equal EID_{50} and HA concentrations, both yielded similar quantities of HA but the former (UP) produced considerably less infectious virus. As NIHA could be produced in MK tissue culture with UP seeds the following experiment was carried out to examine the possibility of producing NIHA with seeds partially inactivated by heat.

Standard PR8 allantoic fluid was diluted 1 : 10 in Parker's medium and this suspension was added to empty tissue culture tubes in 2 ml. amounts. The tubes were then incubated at

35° C. and at intervals the fluids from 3 tubes were harvested and titrated for infectivity and haemagglutinating activity.

TABLE 19. The effect of incubation at 35° C. on the egg infectivity and haemagglutinating activity of PR8 influenza virus.

Incubation hrs.	Titres per ml. log.		
	EID_{50}	HA	ID/HA
0	9.0	3.0	6.0
18	8.6	2.9	5.7
42	7.0	3.0	4.0
66	6.0	2.7	3.3
90	5.2	2.8	2.4

The titres obtained are shown in Table 19 and from these results it was calculated that during an incubation period of 90 hours at 35° C., the infectivity of the virus suspension decreased by 3.8 log giving a value of 0.042 log/hour.

The heat-inactivated fluids described in Table 19 were then inoculated in 0.2 ml. amounts into monkey kidney cultures and the growth curves thus obtained are shown in Fig. 21. In contrast to the results obtained with egg-passaged (UP) fluids and tissue culture-passaged (TCUP) fluids (Figs. 16 and 17), there was no evidence of NIHA production, the ID/HA ratios being of the order of 10^6 in all cases. As was the case with the UP fluids, however, the CPE produced decreased with the amount of virus present in the inoculum.

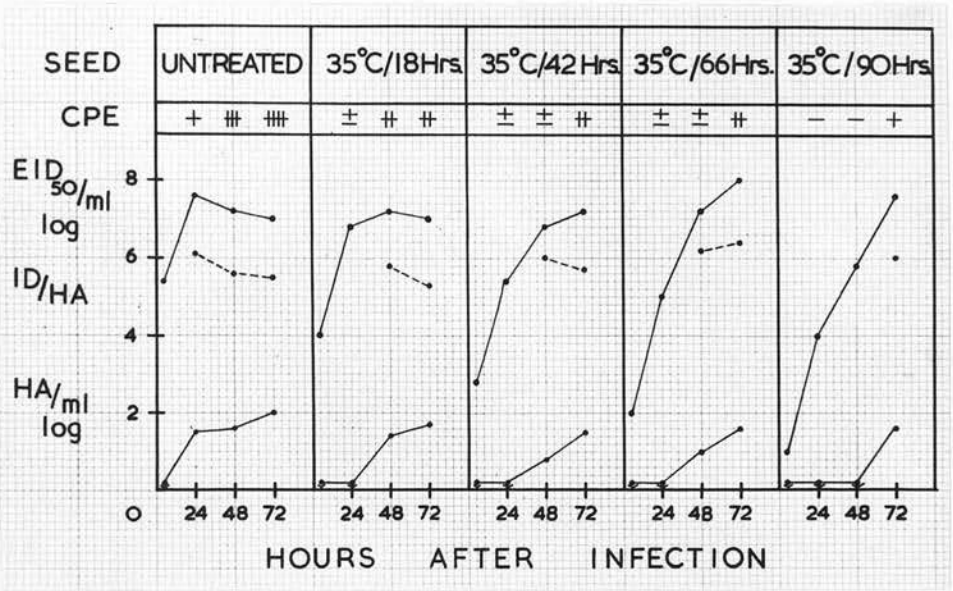

Figure 21.
The development of infectivity, haemagglutinating activity and CPE in monkey kidney cultures after inoculation with 0.2 ml. of standard virus partially inactivated by incubation at 35° C.

The effect of ultraviolet irradiation on the growth of the virus in monkey kidney cultures was also investigated and the results will be described in the section on interference.

Adsorption of influenza virus to monkey kidney cells.

In preliminary experiments carried out prior to the serial passage experiments it was found that adsorption time did not affect the growth of the virus in monkey kidney cells. A further investigation of the conditions associated with the adsorption of PR8 virus to monkey kidney cells was carried out. Some of these experiments involved the use of RDE which has been shown to prevent the infection of the chick embryo by influenza virus by virtue of modification of the cell receptors (Stone, 1947). It was hoped to obtain some idea of the amount of virus taking part in the infection.

Experiment 30.

Adsorption of PR8 virus to monolayers and suspensions of monkey kidney cells.

Undiluted PR8 virus in 0.2 ml. amounts was added either to tubes containing monolayers of monkey kidney cells or to tubes containing 2 ml. of a suspension of approx. 18×10^6 cells/ml. in Parker's medium. At 0, 1 and 2 hours after infection the medium was removed from the monolayer cultures and titrated for infectivity and haemagglutinating activity; at the same intervals, 3 tubes containing suspended cells were centrifuged at 2000 r.p.m. for 20 minutes and the supernates were titrated.

The results, which are shown in Table 20, did not show any significant reduction of the virus after addition to the cells.

TABLE 20. The infectivity and haemagglutinating activity of PR8 influenza virus after addition to monolayers and suspension of monkey kidney cells.

HOURS	VIRUS TITRES PER ML. LOG.			
	Monolayers		Suspensions	
	EID_{50}	HA	EID_{50}	HA
0	7.8	2.4	7.4	2.1
1	7.6	2.3	7.6	2.1
2	8.0	2.3	8.4	2.2

Experiment 31.

Adsorption of PR8 virus to cell monolayers.

PR8 virus was diluted 1 : 10 and 1 ml. of this was added to tubes containing monkey kidney monolayers and to tubes containing no cells; all tubes were incubated at 35° C. At $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2 and 24 hours the fluids were harvested and the tubes washed three times with 1 ml. Parker's medium. The pooled fluids and washings were then titrated for infectivity and haemagglutinating activity and the results are shown in Table 21.

TABLE 21. The infectivity and haemagglutinating activity of PR8 influenza virus after addition to monolayers of monkey kidney cells.

HOURS	VIRUS TITRES PER ML. LOG.			
	With cells		Without cells	
	EID_{50}	HA	EID_{50}	HA
0	8.4	2.3	8.4	2.3
$\frac{1}{2}$	8.4	2.3	8.0	2.3
1	8.6	2.5	8.6	2.3
$1\frac{1}{2}$	9.2	2.4	8.6	2.4
2	8.6	2.4	9.0	2.4
24	7.8	2.6	7.0	2.5

Again no significant reduction in virus titre could be detected by the methods used, although a slight decrease in infectivity due to heat inactivation could be measured over the 24 hour period. A similar result was obtained using bovine embryo kidney cells.

Experiment 32.

Adsorption of PR8 virus to bottle cultures of monkey kidney cells.

The medium was removed from the cultures which contained over 10^6 cells per bottle and 0.5 ml. of a 10^{-1} or 10^{-2} dilution of PR8 virus was added. After 2 hours at 35° C. the original inoculum and the medium harvested from the cells were titrated.

TABLE 22. The infectivity and haemagglutinating activity of PR8 influenza virus after addition to bottle cultures of monkey kidney cells.

DILUTION	INOCULUM		AFTER 2 HR. AT 35° C.			
			With cells		Without cells	
	EID_{50}	HA	EID_{50}	HA	EID_{50}	HA
1 : 10	8.4	1.9	8.0	1.7	7.4	1.4
1 : 100	6.8	-	6.4	-	7.0	-

The results are shown in Table 22 and once more fail to demonstrate the amount of virus adsorbed to the cells.

Experiment 33.

The effect of RDE on the growth of PR8 virus in monkey kidney tissue culture.

The medium was removed from the tube cultures of monkey

kidney cells. To half of these 1 ml. RDE was added and to the remainder 1 ml. Parker's medium; the tubes were left at 35° C. for 2 hours. After this, all the tubes were washed once with 2 per cent. citrate saline and once with Parker's medium, and 1.8 ml. medium and 0.2 ml. undiluted PR8 were then added. After 2 hours the cells were washed twice with Parker's medium. Finally, 2 ml. Parker's medium was added and the cultures were incubated. At $2\frac{1}{2}$ and 24 hours after infection, fluids were harvested from 3 tubes in each group and these were titrated for infectivity and haemagglutinating activity.

TABLE 23. The effect of RDE on the growth of PR8 influenza virus in monkey kidney cells.

HOURS	VIRUS TITRES PER ML. LOG.			
	RDE-treated		Control	
	EID_{50}	HA	EID_{50}	HA
$2\frac{1}{2}$	5.2	-	5.6	-
23	7.0	2.0	7.6	2.3

The titres are shown in Table 23. It was not possible to detect any significant effect of RDE on the multiplication of PR8 in monkey kidney cells although the HA did appear slightly lower in the RDE-treated cultures.

In a similar experiment carried out over the first 2 hours after infection, no difference in adsorption by RDE treated and untreated cells was detected (Table 24), but, as has become evident during this study, it was not possible to measure the amount of virus adsorbed by the methods available and this result

cannot be considered conclusive. It does appear, however, that the treatment of monkey kidney cells with RDE prior to infection with PR8 virus had little or no effect on the actual multiplication of the virus.

TABLE 24. The effect of RDE on the growth of PR8 influenza virus in monkey kidney cells.

HOURS	VIRUS TITRES PER ML. LOG.			
	RDE-treated		Control	
	EID_{50}	HA	EID_{50}	HA
0	7.4	2.3	7.2	2.3
1	7.2	2.3	7.0	2.1
2	7.2	2.3	7.2	2.1

Survival of the cells after infection with influenza virus.

In experiments with NDV in HeLa cells it was found that more cells survived infection with a large inoculum than with a small inoculum (see Experiments 9 and 10). Such an effect was, however, not obvious with influenza-infected monkey kidney cells and some experiments were therefore carried out with this system to investigate the survival properties of these cells.

An attempt to subculture cells after treatment with dilute PR8 influenza virus was unsuccessful, and throughout the growth curve experiments the survival of monkey kidney cells after infection with influenza virus was never so striking as with HeLa cells after NDV infection. With an undiluted inoculum of PR8 virus it was not unusual for all the monkey kidney cells to be destroyed after 72 hours; it is possible that the HeLa cells were protected by the presence of serum in the medium, whereas the monkey kidney cells were maintained in serum-free Parker's medium 199.

It had been noticed (see Experiment 25) that cells survived an infection with egg-passaged UP 2 and UP 3 better than an infection with standard PR8. An experiment was therefore designed to investigate whether any protective mechanism had been conferred on the cells after infection with these egg-passaged seeds.

Experiment 34.

The effect of previous treatment of monkey kidney cells with UP allantoic fluid on the growth of standard PR8 influenza virus.

Monkey kidney cultures were inoculated with either UP 2 or UP 3 allantoic fluid and after 72 hours' incubation a challenge inoculum of standard PR8 virus was added. The experimental scheme is shown in Table 25.

TABLE 25. Experimental scheme to ascertain the effect of previous treatment of monkey kidney cells with UP fluid on the growth of standard PR8 influenza virus.

TUBES	INITIAL TREATMENT		AFTER 72 HOURS		
6		Washed after 2 hrs.	All washed twice	PR8	Washed after 2 hrs.
6	UP 2				
6	UP 2	2 ml. Parker added	1.8 ml. Parker added	- PR8	2 ml. Parker
6	UP 3				
6	UP 3	Incubated 35° C.		-	35°C.

At $2\frac{1}{2}$ hours and 24 hours after addition of the challenge dose, the fluids from three tubes from each group were harvested and titrated for infectivity and haemagglutinating activity. The medium was then changed and at 48 hours the fluids were again examined for infectivity and haemagglutinating activity. The cells were also examined for CPE.

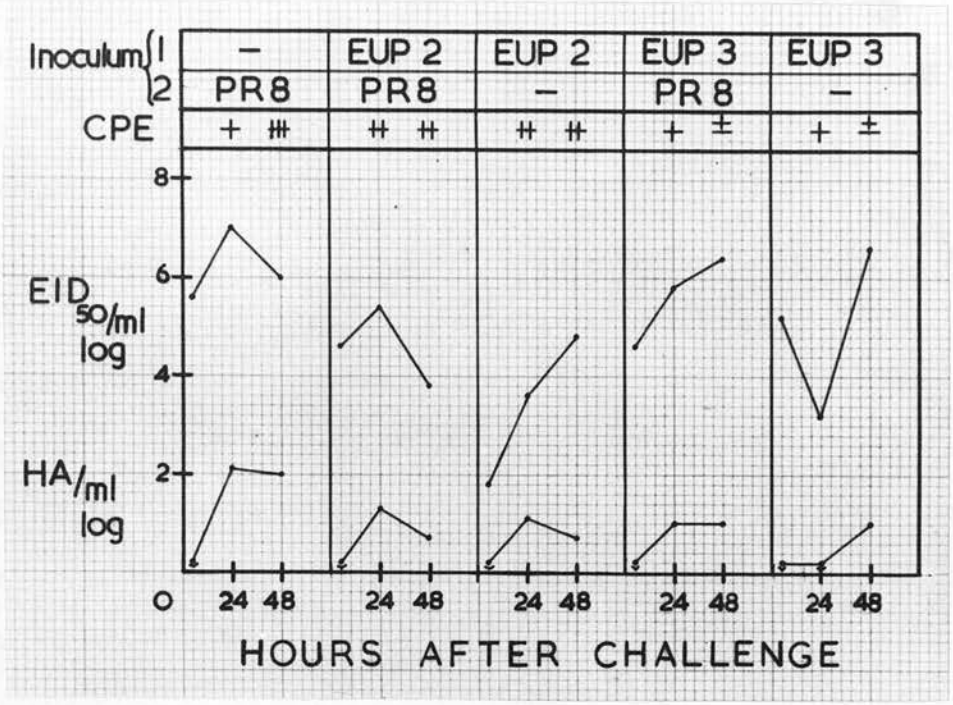

Figure 22.
The effect of previous treatment of monkey kidney cells with UP allantoic fluid on the growth of standard PR8 influenza virus.

The reaction of the cells after addition of the challenge virus is shown in Figure 22. These results are difficult to assess. At first sight it appears that treatment of the cells with UP 2 had significantly affected the multiplication of the challenge dose as both the infectivity and haemagglutination titres achieved were considerably lower than those of the control; cellular changes were also less evident. But if it is borne in mind that some of the cells were damaged by UP 2 before addition of the challenge inoculum and are thus excluded from the experiment, the results appear less conclusive. In spite of this, it was apparent that an interference phenomenon was playing a part in the infectious process. When UP 3 treated cells were

infected with standard PR8, the production of infective and haemagglutinating virus was again reduced, indicating interference by UP 3. Previous treatment of the cells with UP 3 had reduced considerably the CPE of the challenge virus.

These results were not altogether surprising as incomplete virus, prepared by serial allantoic passage of high concentrations of virus, has been shown to induce interference with the growth of the influenza virus in the chick embryo (von Magnus, 1954) and more recently Paucker and Henle (1958) have found that similar preparations of incomplete virus, treated with ultraviolet light, were good interfering agents.

Interference experiments.

The interfering capacity of the influenza virus has been studied extensively. Most of these studies have been carried out in embryonated eggs but more recently tissue cultures have been included in the investigations (Tyrrell, 1955; Isaacs, 1959; Wesslén et al., 1959). Some experiments were therefore included in the present investigation to determine whether or not the interference phenomenon could be produced in the monkey kidney cell PR8 system.

All the experiments entailed investigation for homologous interference. Three types of inactivated virus preparations were used in these studies; ultraviolet (UV) treated virus and seeds inactivated by heat at 56° C. and 35° C. In all cases the virus to be used as interfering agent was concentrated by one adsorption on to chicken erythrocytes and the challenge inoculum consisted of 0.2 ml. of a diluted virus suspension containing approximately 10^6 EID_{50}/ml.

During the preliminary experiments, evidence was obtained that virus heated at 56° C. was a better interfering agent than UV irradiated virus; however, on investigating this, it was found that the interfering activity of the UV virus had been completely removed by irradiation due to the unprotective nature of serum-free Parker's medium, which was used as a diluent. In further experiments, all three interfering agents were shown to produce effective interference. One of the preliminary experiments is described below.

Experiment 35.

The interfering effect of UV inactivated virus, heat inactivated virus and the corresponding interferon preparations on the growth of standard PR8 virus in monkey kidney cells.

(a) Preparation of inactivated virus. PR8 allantoic fluid was diluted 1 : 10 in Parker's medium after concentration by adsorption on to chicken erythrocytes. Part of this fluid was irradiated by UV light for 60 sec. x 3 and another 20 ml. was kept at 56° C. for 1 hour. These fluids constituted the interfering agents.

(b) Addition of inactivated virus to monkey kidney cells. After removing the medium from the cultures they were divided into three groups:

group A received 1 ml. Parker's medium;

group B " " UV virus;

group C " " 56° C. virus.

The tubes were then incubated at 35° C. for 3 hours in the roller drum, after which they were washed twice with Parker's medium. Fresh medium was added and the cultures were incubated overnight (18 hours) in the roller drum.

(c) Addition of challenge virus. The fluids were harvested and after centrifugation in the Spinco at 3,500 r.p.m. (approx. 1000 G) for 20 minutes they were stored at 4° C. for use in the "interferon" experiments.

PR8 virus diluted 10^{-4} was then added to the cultures in 0.2 ml. amounts and after washing twice after 2 hours they were

sampled at 2½ hours, 21, 45 and 70 hours, and tested for infectivity and haemagglutinating activity. The cells were also examined for CPE. The results are combined with those of (d) in Figure 23.

(d) To test the activity of interferon preparations. The medium was removed from the uninfected monkey kidney cultures and 1 ml. of the interferon preparation was added to each of 3 groups. The tubes were incubated in the roller drum at 35° C. for 24 hours. The fluids were then removed and discarded and 0.2 ml. PR8 virus diluted 10^{-4} in 1.8 ml. Parker was added; after incubation at 35° C. for 2 hours, the cells were washed twice and at intervals of 2½, 23, 47 and 70 hours after inoculation of challenge the fluids were harvested from three tubes from each group. These were titrated for infectivity and haemagglutinating activity.

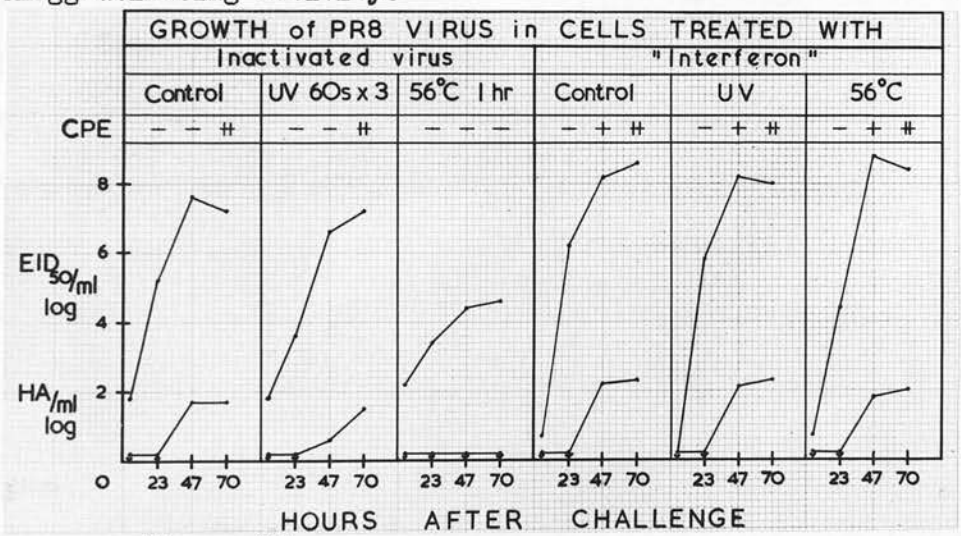

Figure 23.
The interfering effect of UV-inactivated virus, heat-inactivated virus and the corresponding interferon preparations on the growth of standard PR8 virus in monkey kidney cells.

On examining the combined results which are shown in Figure 23, it was seen that although the 56° C. virus interfered considerably with the multiplication of the challenge and the UV preparation to a small extent, the effect of the interferon preparations was not detectable.

Experiment 36.

The effect of interferon prepared from virus heated for varying times on the growth of standard PR8 virus in monkey kidney cells.

In a further experiment of this type, in which a more concentrated suspension of virus was treated at 56° C. for ½, 1, 2 and 4 hours to form the interfering agents, evidence for the production of an interferon-like substance was obtained. The most efficient interfering agent was that treated at 56° C. for 1 hour. It may be seen from Figure 24 that it was also this interferon preparation which produced the most noticeable reduction in growth of the challenge virus. In these experiments it was evident that the interferon-like effect was most noticable 24 hours after addition of the challenge inoculum, after which time it tailed off.

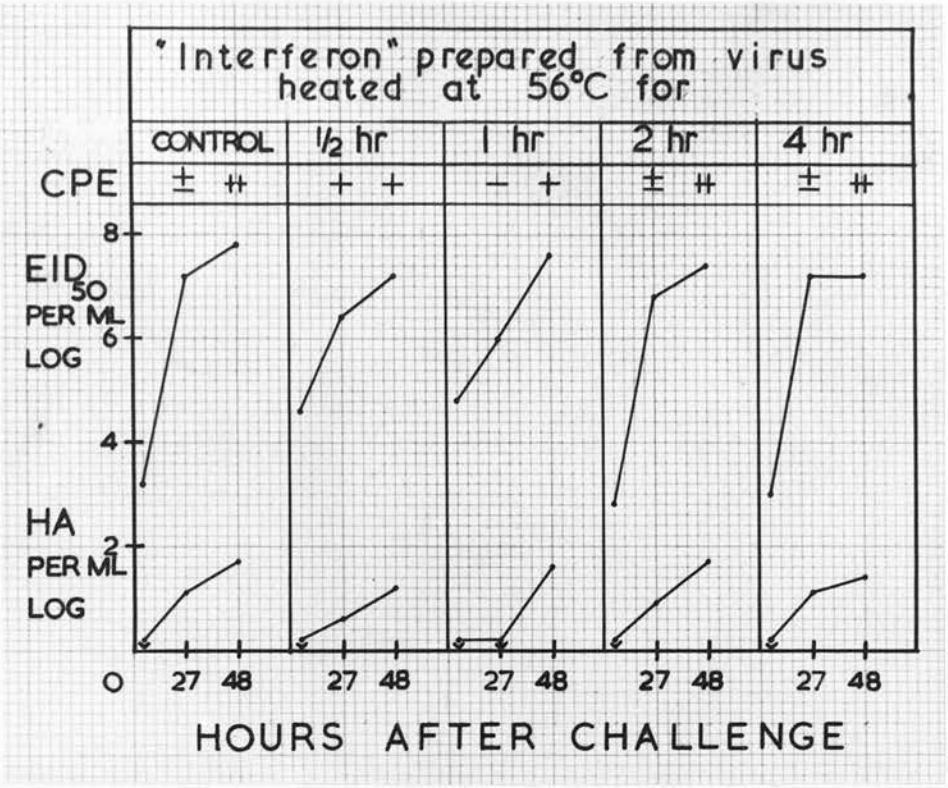

Figure 24.
The effect of interferon prepared from virus heated for varying times on the growth of standard PR8 virus in monkey kidney cells.

A typical experiment where UV virus was shown to act as an excellent interfering agent is described below.

Experiment 37.

The interfering activity of UV inactivated PR8 virus on the growth of standard PR8 virus in monkey kidney cells.

The seed virus, after concentration by adsorption on chicken erythrocytes, was treated with UV light for varying lengths of time, i.e. from 30 seconds x 2 to 120 seconds x 2. These fluids were then added in 0.5 ml. amounts to groups of monkey kidney cultures from which the medium had been removed. The cultures were incubated for 4 hours at 35° C., after which the medium was removed and the cells were washed twice with

Parker's medium. Parker's medium was then added to all tubes in 1.8 ml. amounts, after which half of each group received the challenge inoculum. All cultures were washed after 2 hours' further incubation and 2 ml. fresh medium was added; at intervals the cells were examined for CPE and the fluids from 3 tubes from each group were harvested and titrated for infectivity and haemagglutinating activity.

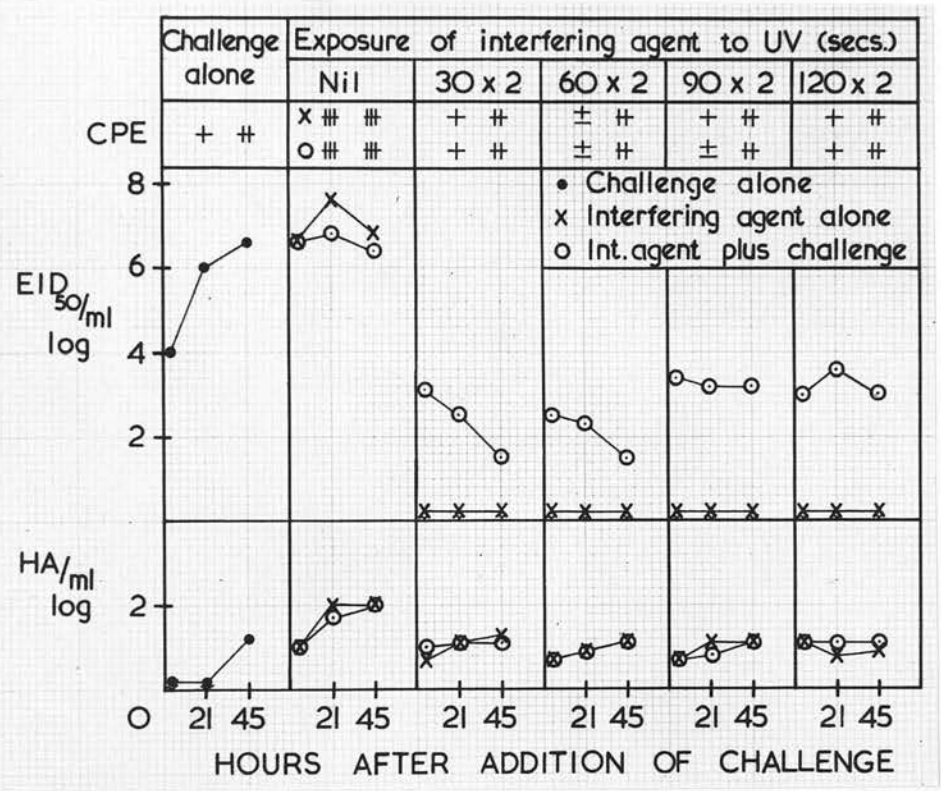

Figure 25.

The interfering activity of UV-irradiated PR8 virus on the growth of standard PR8 virus in monkey kidney cells.

The results are shown in Figure 25. In this experiment all the inactivated fluids induced very striking interference and it appears that, with continued irradiation of the virus, there was a gradual decrease in interfering ability. As with

heated virus, there appeared to be an optimum of inactivation capable of producing the most striking interference.

There was no definite evidence of the production of incomplete virus by UV-inactivated fluids. Definite cytopathic changes were produced by these fluids without detectable multiplication of infectious virus. This was investigated further and will be described in the next section.

The cytopathic effect of influenza virus.

The cytopathic changes produced in monkey kidney tissue cultures by influenza A, B and C viruses have already been described and have been found to be similar (Mogabgab et al., 1955; Takemoto et al., 1955). The general changes observed in the cultures included granulation, rounding of the cells, and pyknosis and disintegration of the nuclei. However, until the recent description by Negroni and Tyrrell (1959) of the morphological changes produced in kidney epithelial cells by influenza A, the process has not been studied in detail. A more specialised study was carried out with the aid of the fluorescent acridine orange technique and it was possible to determine specific nucleic acid changes associated with influenza virus infection (Anderson et al., 1959).

The investigations mentioned above do not provide conclusive evidence of the changes induced in tissue cultures by the PR8 strain of influenza A virus. Some experiments were therefore carried out in which PR8-infected monkey kidney and bovine embryo kidney cells were stained either with haematoxylin and eosin, or with acridine orange.

Experiment 38.

The CPE of the PR8 strain of influenza virus examined by the haematoxylin and eosin staining method and by the acridine orange technique.

Coverslip cultures of monkey kidney and bovine embryo kidney cells were infected with PR8 influenza virus. After

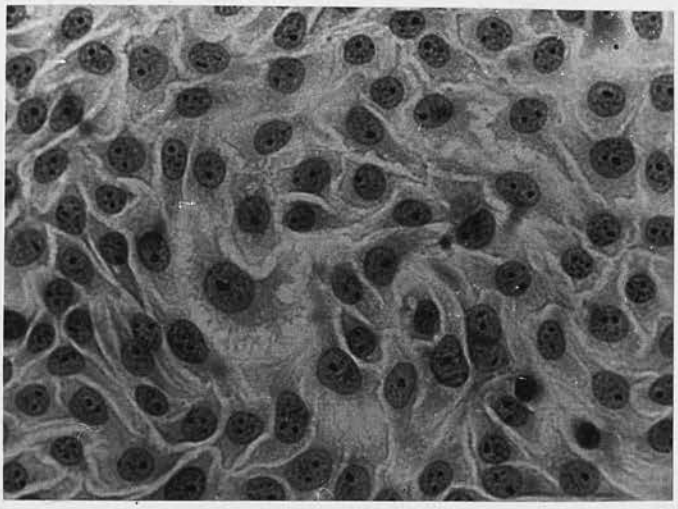

Figure 26.

Uninfected monkey kidney cells.
Haematoxylin and eosin. x 360.

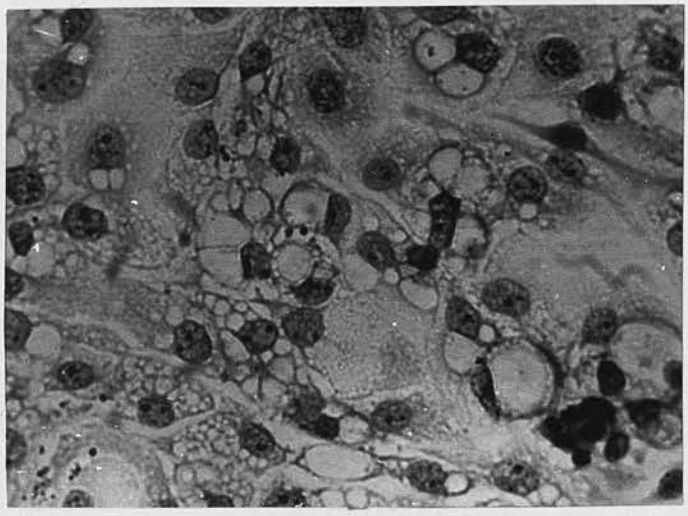

Figure 27.

Monkey kidney cells 48 hrs. after infection with PR8 influenza virus.
Haematoxylin and eosin. x 360.

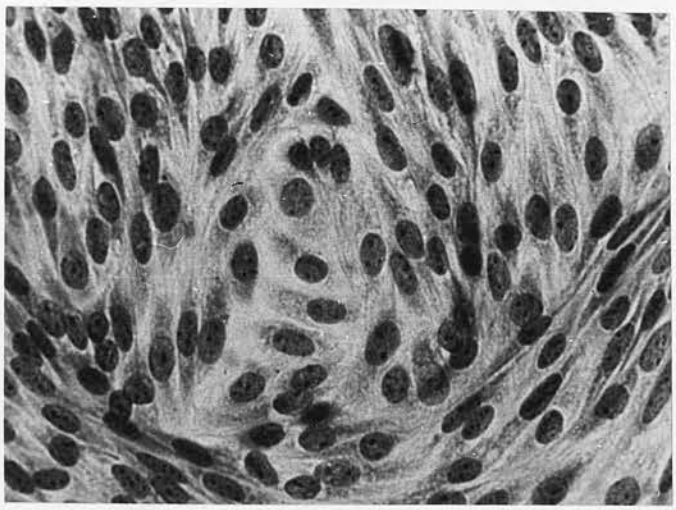

Figure 28.

Uninfected bovine embryo kidney cells.
Haematoxylin and eosin. x 360.

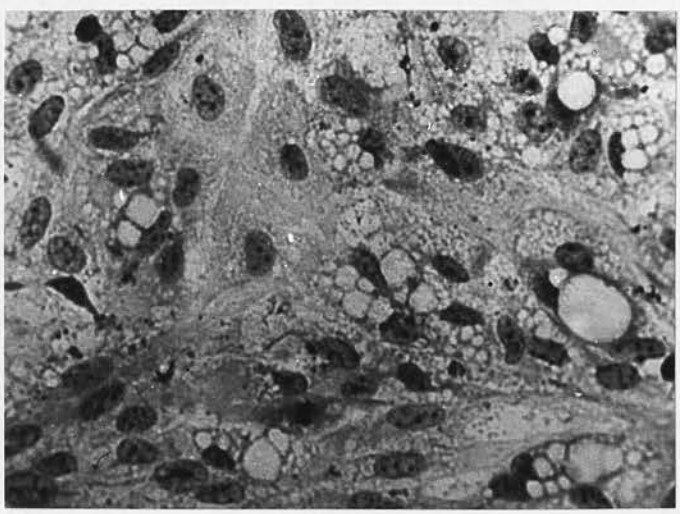

Figure 29.

Bovine embryo kidney cells 48 hrs. after infection with PR8 influenza virus.
Haematoxylin and eosin. x 360.

48 hours the cells were fixed and stained.

Haematoxylin and eosin. The general appearance of the cells was similar to that previously described by other workers and may be seen in Figures 27 and 29. The most obvious change in the cytoplasm was the development of vacuoles; these were visible at 24 hours after infection, and at 72 hours heavy vacuolation was seen throughout the entire cell monolayer. This effect is evidently quite characteristic for the PR8 strain of influenza and has been previously observed in swine kidney cells (Haas and Wulff, 1957). The development of intranuclear changes in infected cells was observed; many nuclei appeared to be divided up by basophilic septa and can be distinguished in the accompanying photomicrographs.

Acridine orange. The morphological changes associated with influenza virus infection as seen with the acridine orange technique have been quite extensively described by Anderson et al. (1959) but these descriptions apply almost solely to the Mel and WS strains of the virus. When PR8 infected monkey kidney and bovine kidney cultures were examined by the acridine orange technique during the present investigations, nuclear changes were detected but these were not so pronounced as with Mel and WS. This led to the idea, previously conceived by Dr. Niven (personal communication) that the nucleic acid changes produced in infected cells depended on the strain of virus used. Some comparative experiments were therefore carried out in collaboration with Dr. Niven to confirm this observation.

Experiment 39.

The detection of strain differences by means of the acridine orange technique.

In these experiments coverslip cultures of bovine embryo kidney cells containing approximately 300,000 cells per tube received about 1 HA unit of either WS, Mel or PR8 influenza virus. The cultures were incubated overnight at 35.5° C.

The appearances of the cells, fixed and stained 18 hours after infection, were as follows:

Uninfected cells contained oval or circular nuclei with two or more nucleoli; the associated chromatin and granules of precipitated chromatin fluoresced a greenish-yellow colour indicating the presence of DNA; the nuclear membrane showed up as a continuous line with an occasional small chromatin granule attached to it. In the cytoplasm, the red fluorescing RNA exhibited a diffuse fine granularity extending uniformly almost to the limit of the cell and closely surrounding the nucleus (see Figure 30).

Infected cells. Changes following infection appeared both in the nucleus and the cytoplasm.

WS and Mel. Enlargement of the nucleoli was accompanied by the development of a diffuse red colour which entirely filled the nucleus (see Figure 31). At first the chromatin granules and the nucleoli could still be distinguished but as the red fluorescence deepened, all the other structures became invisible except the associated chromatin of the nucleoli which remained distinguishable as yellow dots; the fully

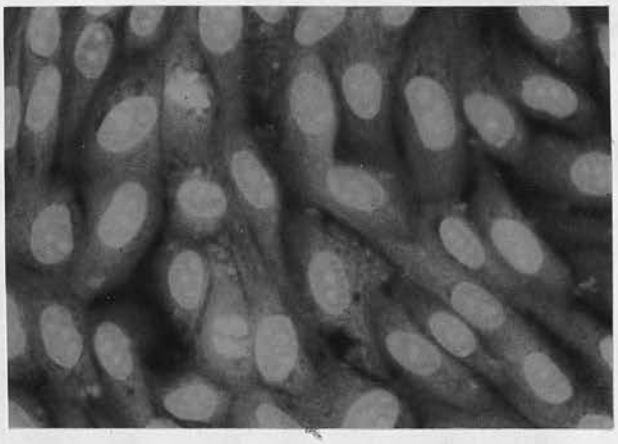

Figure 30.

Uninfected bovine embryo kidney cells.
Acridine orange. x 225.

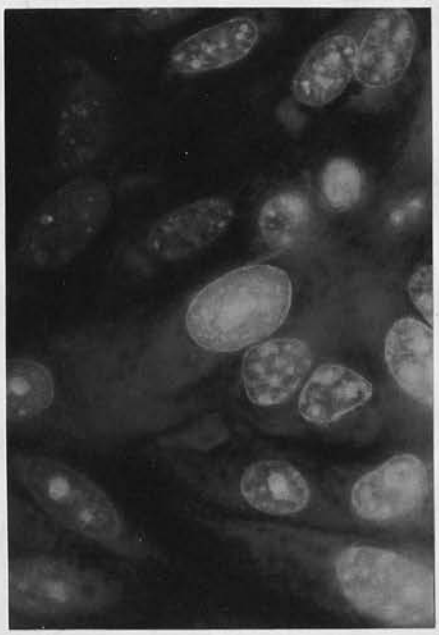

Figure 31.

Bovine embryo kidney cells
18 hrs. after infection with
WS influenza virus.
Acridine orange. x 325.

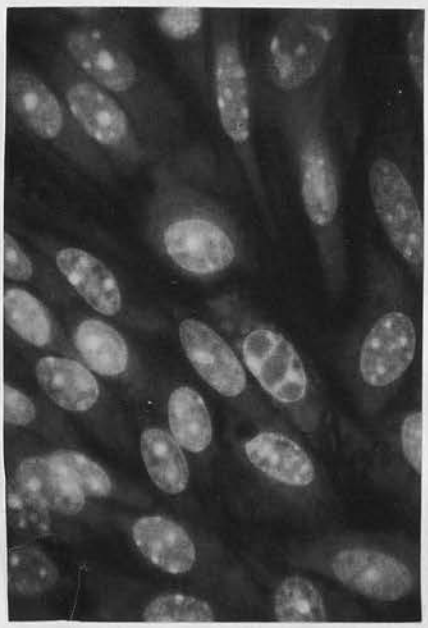

Figure 32.

Bovine embryo kidney cells
18 hrs. after infection with
PR8 influenza virus.
Acridine orange. x 325.

developed nuclear RNA was strikingly homogeneous. The outline of the nuclear membrane became indistinct and acquired a finely beaded appearance suggestive of breaks in continuity. Cytoplasmic changes were apparent at first only as an increased intensity of red RNA fluorescence and were not always associated with the development of intranuclear RNA. Occasionally tufts of RNA were seen extending from one or both poles of the nucleus. In WS infected cells the intranuclear RNA fluorescence was very distinct but only a proportion of the cells in the culture showed it at a given time. In MEL infected cells the intranuclear fluorescence was never so conspicuous, but the number of cells showing alterations was greater than in WS infected cells. It was noted at this stage when the intranuclear RNA staining was so intense that the Coon's reaction was generally negative in the nucleus, but positive in the cytoplasm. Nudei with less pronounced RNA accumulation gave the most intensely positive Coon's result.

PR8: In PR8 infected cultures, flooding of the nucleus with RNA, as occurs with Mel and WS, was not found but it was possible to find nuclei which showed slight diffuse RNA tinging. The most striking and constant feature was the development of nuclear vacuoles separated by chromatin strands which gave a greenish fluorescence (see Figure 32). These were not dissimilar in appearance to the nuclei seen in haematoxylin stained PR8 infected cells (Figures 27 and 29). Cytoplasmic vacuolation associated with focal accumulation of RNA was also seen.

Experiment 40.

An attempt to detect incomplete virus production by means of the acridine orange technique.

It was hoped that as specific cellular changes due to PR8 virus were detectable by the acridine orange staining technique it might be possible to detect some abnormal changes in nucleic acid production when incomplete virus was formed.

Bovine embryo kidney cells were inoculated with PR8 allantoic fluid which had undergone two undiluted passages in eggs. This EUP 2 was used undiluted and 10^{-2}. At intervals the cells were fixed and stained with acridine orange. The preparations were examined in the fluorescent microscope for abnormal changes which might be associated with incomplete virus production. Although some unusual nuclear appearances were detected they could not be correlated with any irregular synthetic process. The typical nuclear and cytoplasmic changes previously described for PR8 were observed.

Correlation of CPE with multiplication of the virus.

The production of cytopathic changes by influenza virus has been reported to be associated with haemagglutinin production (Takemoto et al., 1955) and although our findings in the present study do not contradict this observation they do suggest that the amount of CPE production is dependent on the amount of infectious virus present in the inoculum. This was demonstrated in the following experiment:

Experiment 41.

Relation between infectivity of inoculum and cytopathic effect in monkey kidney cells.

Monkey kidney cultures were inoculated with standard virus (undiluted 10^{-1}, 10^{-2}), EUP 1 (undiluted 10^{-1}) and EUP 2 (undiluted) and incubated for 24 hours. The fluids were harvested and titrated for infectivity and haemagglutinating activity and the cells were examined for CPE.

TABLE 26. Relation between infectivity of inoculum and cytopathic effect in monkey kidney cells.

INOCULUM			DAYS AFTER INOCULATION				
			1		3		
Type	EID_{50}	HA	CPE	HA	CPE	EID_{50}	HA
St UD	10.2	4.2	++	2.8	+++	7.8	3.2
1 : 10	9.2	3.2	++	2.3	+++	6.8	2.6
1 : 100	8.2	2.2	+	1.8	+	7.4	2.1
UP 1 UD	9.2	4.2	++	2.8	+++	7.0	3.0
1 : 10	8.2	3.2	++	1.9	++	7.0	2.1
UP 2 UD	8.4	4.0	+	2.7	+	5.4	3.0

St = standard virus; UD = undiluted;

UP 1 = allantoic fluid which has undergone one undiluted passage in eggs;

UP 2 = allantoic fluid which has undergone two undiluted passages in eggs.

The results are shown in Table 26. When comparing the CPE of these different seeds it is apparent that the CPE was due in most part to the presence of infectious virus in the inoculum

and therefore would seem to be closely related to the virus particle. This is not to say, however, that CPE is not related in time to haemagglutinin production, but the extent of cellular damage appears to be associated with the presence of infectious virus.

Experiment 42.

The CPE of untreated and UV-irradiated PR8 influenza virus in monkey kidney cells.

A further attempt to correlate the CPE of the virus with some other manifestation of infection was made by studying the CPE induced by untreated and Uv-irradiated standard virus and concentrates of standard virus.

Standard PR8 virus was concentrated by two adsorptions on to chicken erythrocytes and 3 fluids were thus obtained: standard unadsorbed virus, EB 1 (1st concentrate), and EB 2 (2nd concentrate). Each of these fluids was divided into 3 parts and these were subjected either to UV irradiation for 30 seconds x 2, 120 seconds x 2 or to no irradiation. After titration for infectivity and haemagglutination the fluids (treated and untreated) were then inoculated in 0.2 ml. amounts into monkey kidney cells. At intervals the culture fluids were harvested from the cells and titrated for infectivity and haemagglutinating activity; the cells were examined for CPE.

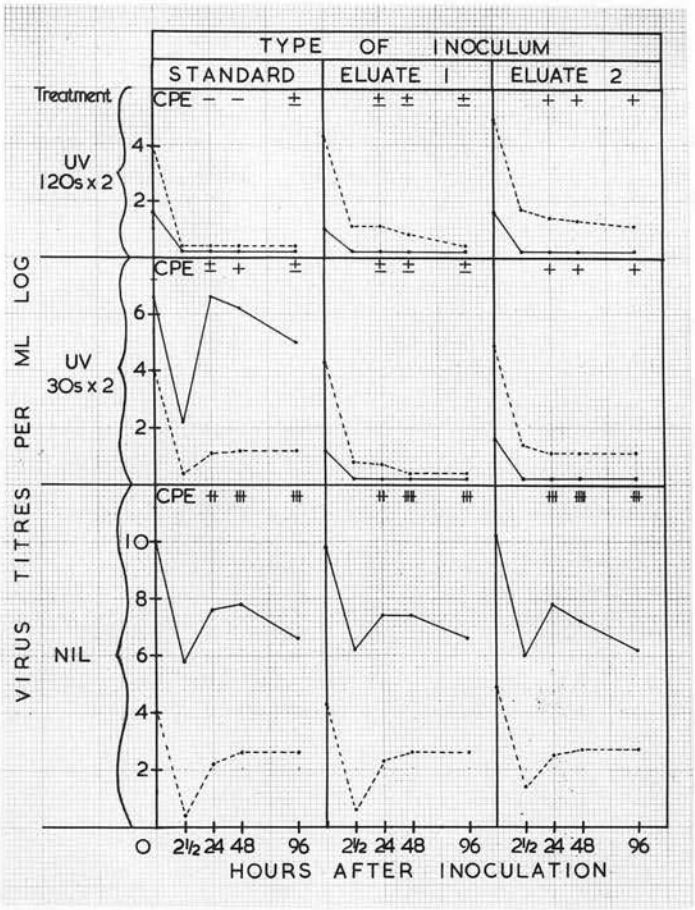

Figure 33.

The CPE and growth of untreated and UV-irradiated PR8 influenza virus in monkey kidney cultures.

The results are shown in Figure 33. The untreated fluids showed concomitant CPE and increase in egg infective and haemagglutinating virus. The partially irradiated standard virus, on the other hand, showed considerable multiplication with little damage to the cells; the cellular changes were in fact similar to those observed with irradiated EB 1 and EB 2, where there was no detectable multiplication. It appears, therefore, that UV irradiation had partly removed the cytopathogenic property of the virus without damaging its capacity to multiply.

So far, however, there is no evidence that the CPE of influenza virus can be separated from the other properties and

it must be concluded that, under conditions of normal multiplication cytopathic changes occur as the virus multiplies. The extent of these changes is related to the amount of virus in the inoculum and not to the amount liberated into the medium. Under certain conditions, i.e. those giving rise to incomplete virus formation, the cytopathic changes are considerably less; this could be explained by the fact that incomplete virus preparations are known to be less toxic than standard preparations.

The use of the fluorescent antibody technique in an investigation of the multiplication of influenza virus.

Experiment 43.- Preliminary experiment.

The detection of specific fluorescence in bovine embryo kidney cells infected with the PR8 and Asian strains of influenza virus.

The preliminary investigations using this technique were concerned with the infection of bovine embryo kidney cells with PR8 and an Asian strain (VI, 160). These viruses were added to coverslip cultures as 1 : 100 dilutions of allantoic fluid and 18 and 24 hours after infection the cells were fixed and treated with convalescent or acute human anti-influenza A serum, followed by rhodamine labelled rabbit anti-human gamma-globulin serum. Examination of these preparations in the fluorescent microscope showed no specific fluorescence in the uninfected controls and in those cells treated with acute antiserum. In the preparations treated with convalescent serum, however, nuclear staining was easily visible with both viruses at 18 and 24 hours; in the case of PR8 the majority of cells showed reddish fluorescence which by 24 hours had become defined into aggregates towards the border of the nucleus, whereas the staining in the Asian infected cells was more diffuse and almost completely filled the nucleus.

Having ascertained that specific fluorescence could be observed under the conditions of our experiments, the investigations were continued.

Experiment 44.

Growth curve of PR8 influenza virus in bovine embryo kidney cells examined by titration methods and the fluorescent antibody technique.

Coverslip cultures of bovine embryo kidney cells were inoculated with 2 ml. of 1 : 100 PR8 virus. After 2 hours the cells were washed twice with Parker's medium; 2 ml. medium was then added and the cultures were incubated. At intervals the fluids were harvested from 4 tubes and were titrated for infectivity and haemagglutinating activity. The coverslips were removed from the tubes and washed and fixed and were stored at -20° C. prior to staining. Staining was carried out with acute or convalescent human anti-influenza A serum followed by rhodamine-labelled rabbit antihuman gamma globulin serum.

TABLE 27. Development of infectivity, haemagglutinating activity and specific fluorescence in bovine embryo kidney cells following inoculation with PR8 influenza virus.

HRS.AFTER INFECTION	VIRUS TITRES PER ML. LOG.			FLUORESCENCE	
	EID_{50}	HA	ID/HA	ACUTE	CONVALESCENT
Inoculum	7.6	1.6	6.0		
2	x			-	-
4	6.0	-	-	-	-
6	6.0	-	-	-	+
8	7.2	0.7	6.5	-	++
10	7.0	1.2	5.8	-	++
12	7.8	1.6	6.2	-	++
24	7.8	1.9	5.9	-	+++

x = not titrated.

The titration results and appearance of the corresponding preparations in the fluorescent microscope are shown in Table 27. Specific fluorescence was first visible at 6 hours and was evident as a dull redness appearing at the edge of the nuclei. By 8 hours about 50 per cent. of the cells showed nuclear fluorescence and also a few nuclear vacuoles which increased in number in later preparations. The "bundles" of nuclear fluorescence became larger and by 12 hours some staining was seen in the cytoplasm. At 24 hours very bright fluorescence was observed in the nuclei and occurred mostly in aggregates just within the nuclear membrane; many cells, however, showed no staining. It was of interest to note that fluorescence was visible in mitosing cells.

On examining the titration results it appears that the first signs of S-antigen in the nucleus at 6 hours were responsible for the significant increase in infectious and haemagglutinating virus in the medium at 8 hours.

Experiment 45.

An attempt to investigate the incomplete cycle of multiplication of PR8 influenza virus by means of titration methods, the fluorescent antibody technique and the acridine orange staining method.

It was not possible, by means of the acridine orange staining technique, to detect significant differences in the nucleic acid changes when an apparently incomplete cycle was taking place within the cells; this was surprising as incomplete virus is known to be deficient in RNA. As incomplete virus is

also known to be deficient in S antigen a comparative study was carried out using the acridine orange technique in parallel with the fluorescent antibody technique. Sera prepared in guinea-pigs to the S antigen of influenza virus were employed followed by labelled anti-guinea-pig gamma globulin.

Three viral inocula (standard PR8 virus diluted 1 : 1000 and 1 : 2 and EUP 2 diluted 1 : 2) were inoculated into bovine embryo kidney cultures in 0.2 ml. amounts. The cells were washed in the usual way after 2 hours. At $2\frac{1}{2}$, 12, 24 and 48 hours after infection, representative cultures were removed from the incubator; coverslip preparations were fixed ready for staining with acridine orange or fluorescent antibody and the fluids were titrated for infectivity and haemagglutinating activity. Cells from parallel tubes which contained no coverslips were scraped down by means of a rubber policeman to form a suspension which was subjected to supersonic vibration; the resulting suspension was titrated for infectivity and haemagglutinating activity.

The results of these titrations and the appearances observed in the fluorescent microscope are presented in Table 28 (Appendix). With the fluorescent antibody technique the undiluted passage virus (EUP 2) gave a pattern very similar to, although slightly weaker than, that observed with the dilute inoculum of standard virus (Figures 34, 35, 36); the nucleus was brightly stained, especially in the neighbourhood of the nuclear membrane, giving a beaded appearance. Practically all the cells were infected. In the case of the concentrated

Bovine embryo kidney cells 48 hrs. after infection with PR8 influenza virus. Treated with guinea-pig anti-S serum and rhodamine-labelled rabbit anti-guinea-pig gammaglobulin serum.

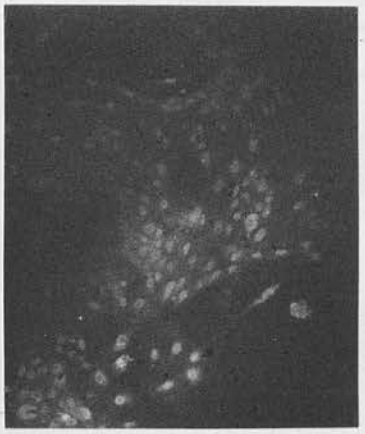

Figure 34.

Standard PR8
diluted 10^{-3}.

x 100

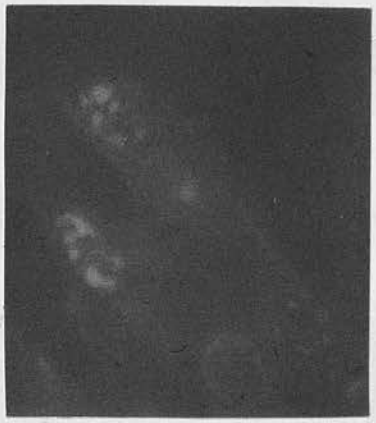

Figure 35.

EUP 2 1 : 2.

x 360

Figure 36.

Standard PR8
diluted 10^{-3}.

x 360

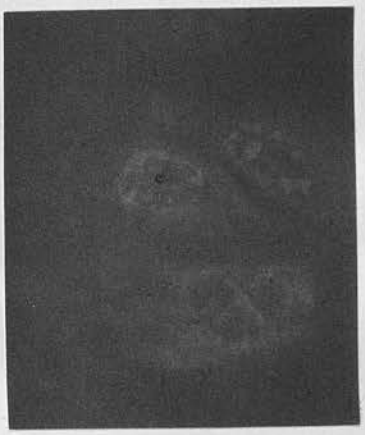

Figure 37.

Standard PR8
diluted 1 : 2.

x 360

inoculum of standard virus, most cells fluoresced but the nuclear staining was considerably weaker than that observed with the dilute inoculum (Figure 37). This was surprising as the titration results did not show evidence of incomplete virus production following infection of the cells with a concentrated inoculum, whereas a considerably lower ID/HA ratio was observed with EUP 2. The intense staining observed with a dilute inoculum can be related to the considerable rise in infectivity and haemagglutinating activity detected in the titrations. The low ID/HA ratios obtained in the cell suspensions were presumably due to the presence of intracellular haemagglutinins released by the supersonic disintegration of the cells.

The acridine orange staining did not entirely complement these results. The typical changes previously described for PR8 in bovine embryo kidney cells were observed with the concentrated inoculum and with EUP 2. With the dilute inoculum, however, few changes were seen, even after 48 hours. These results suggest that the two techniques are detecting different stages in the infectious process, but in both cases a difference was observed in cells infected with large and small doses of virus.

The growth of Asian influenza virus in bovine embryo kidney cells.

It had been hoped that the development of the PR8 virus in bovine embryo kidney cells might be further investigated with the aid of the V and S antigens of this strain, but attempts

to obtain such antigens in a sufficiently pure form were unsuccessful. However, the V and S antigens of an Asian strain were available in a high degree of purity, and antisera prepared in guinea-pigs to these antigens showed no cross reactions in chessboard complement-fixation titrations. Experiments were therefore planned to investigate in bovine embryo kidney cells the intracellular appearance of the S and V antigens with respect to site and time after infection, using fluorescent antibody.

Experiment 46.

The development of infectivity, haemagglutinating activity and specific fluorescence in bovine embryo kidney cells following inoculation with a small dose of an Asian strain of influenza virus.

After removal of the growth medium, Leighton tubes containing almost confluent monolayers of bovine embryo kidney cells were inoculated with 2 ml. of a virus suspension diluted 1 : 1000 in Parker's medium. At the time of infection each tube contained approximately 700,000 cells and as the titre of the virus inoculum was of the order of 10^9 EID_{50}/ml., the multiplicity of infection possible in such a system was approximately 2 EID_{50} units per cell. After addition of the virus the tubes were incubated for one hour at 35° C. The fluid was then removed, the cells were washed three times with 1 ml. amounts of Parker's medium and finally 2 ml. medium was added to the cells before returning them to the incubator. At timed intervals, i.e. 2, 4, 6, 8, 10, 12 and 24 hours after infection, 3 coverslip cultures were removed from the tubes and

dried and fixed prior to staining with fluorescent antibody. At the same intervals the culture fluid was harvested from 2 tubes not containing coverslips; the cells were washed gently three times with Parker's medium and were then scraped down by means of a rubber policeman into 2 ml. of medium; the resulting suspension was subjected to supersonic vibration for 15 minutes. Both culture fluid and supersonic treated cells were titrated for infectivity and haemagglutinating activity.

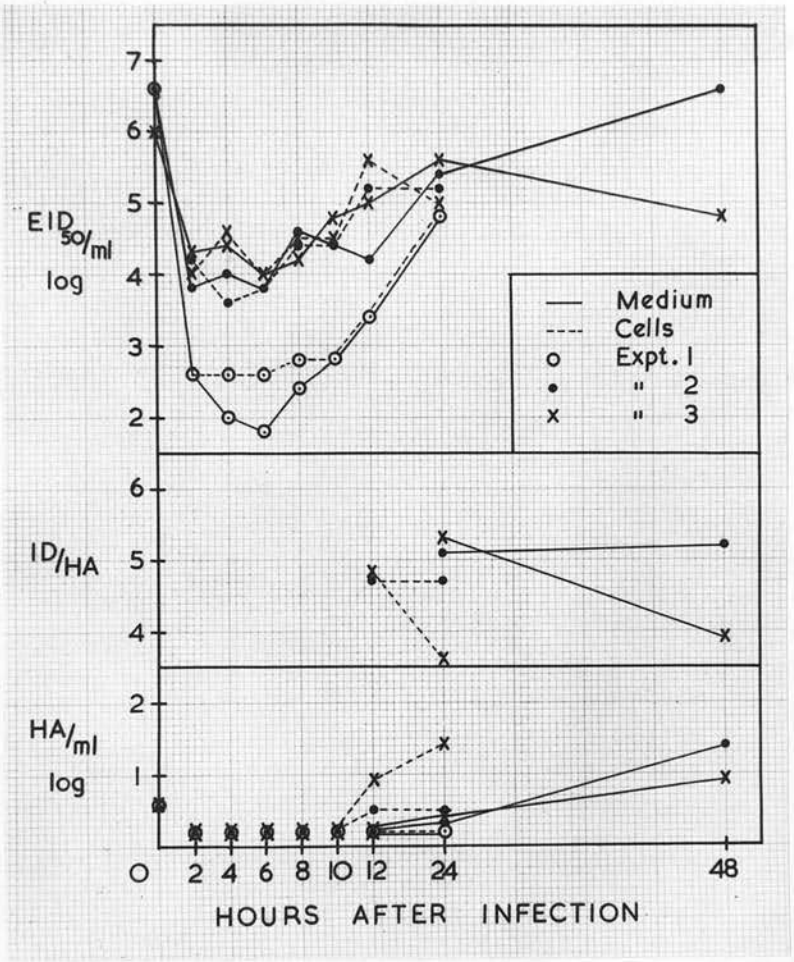

Figure 38.
The development of infectivity and haemagglutinating activity in bovine embryo kidney cells and their nutrient medium following inoculation with a small dose of an Asian strain of influenza virus.

The results of the titrations are shown with the corresponding ID/HA ratios in Figure 38. It will be seen that a significant increase of both infective and haemagglutinating virus occurred.

Fluorescent antibody studies.

Three fixed coverslip cultures from each time interval were treated with specific antisera prepared in guinea-pigs to the whole influenza particle and to the S and V antigens. Labelling was then achieved by means of rhodamine-labelled rabbit anti-guinea-pig gammaglobulin serum and the cells were examined for localisation and intensity of fluorescence. An arbitrary scale was used for scoring the intensities observed and the results are shown in Table 29.

TABLE 29. The development of specific fluorescence in bovine embryo kidney cells infected with a dilute inoculum of Asian influenza virus.

HOURS AFTER INFECTION	ANTISERUM					
	WHOLE		ANTI-S		ANTI-V	
	CYTOPLASM	NUCLEUS	CYTOPLASM	NUCLEUS	CYTOPLASM	NUCLEUS
2	-	-	-	-	-	-
4	-	-	-	-	-	-
6	-	(+)	-	(+)	-	-
8	+	++	(+)	++	+(+)	$+^{x}$
10	+(+)	+++	(+)	+++	++	$+^{x}$
12	++	+++(+)	+	+++	++	$++^{x}$
24	++	+++(+)	+	+++	++	$++^{x}$

Intensity of fluorescence expressed by an arbitrary scale:

(+) = weak intensity; + = fair intensity

++ = moderate intensity, etc.;

x = intensity in the described nuclear foci.

Whole antiserum. No specific orange staining was observed at the intervals 2 hours and 4 hours after infection.

At 6 hours the nucleus in a few cells started to show a scattered, coarse, granular fluorescence of low intensity. In two of the experiments no specific staining was observed in the cytoplasm. In a third, occasional cells which showed nuclear staining also exhibited a faint perinuclear haze. At 8 hours the nuclear staining had gained considerably in intensity and filled the whole nucleus with a coarse granular pattern. The cytoplasmic fluorescence was now definite, but of a lower intensity than that of the nucleus; although staining occurred throughout the cytoplasm it was denser around the nucleus. At 10 hours the nuclei had the same pattern as at 8 hours but of a higher intensity. Several small unstained vacuole-like areas could be observed in the nucleus against the generally fluorescing background. The cytoplasmic fluorescence was at this stage more evenly distributed, showing less perinuclear accumulation than at 8 hours. At 12 hours the nuclei exhibited essentially the same picture of a coarse granular fluorescence with vacuoles, which was seen at 10 hours, although the staining was even more brilliant. The cytoplasmic staining was generally diffuse, although in some cases it was denser along the periphery of the cells. At 24 hours the picture was very much like that at 12 hours, except that peripheral distribution in the cytoplasm was more prominent (Figure 39).

Anti-S serum. At the intervals 2 hours and 4 hours after infection, no specific staining was observed using anti-S serum. At 6 hours occasional cells showed a coarse granular fluorescence of low intensity which occupied the whole nucleus. No fluorescence was observed in the cytoplasm. At 8 hours the nuclear fluorescence was of moderate intensity giving the impression of a coarse, granular reticulum, occupying the whole nucleus. A varying number of small unstained vacuole-like areas were observed very much resembling those noted in the nuclei using whole antiserum. Rare cells showed weak perinuclear staining. At 10 hours the nucleus exhibited bright fluorescence of the same pattern as was described above for 8 hours. In some cells showing nuclear fluorescence, weak diffuse cytoplasmic staining was seen without any conspicuous aggregation. Cells showing cytoplasmic staining without concomitant nuclear staining were never observed. At 12 hours the picture was essentially the same as that at 8 hours, except for the intensity, which in the nucleus had gained a high brilliance. The cytoplasmic intensity was still quite low (Figure 40). At 24 hours the nuclear staining was very much like that of 12 hour preparations but more cells exhibited a diffuse low grade cytoplasmic staining than at 12 hours.

Anti-V serum. As was the case when whole antiserum of the anti-S serum was used, no specific orange staining was observed at 2 and 4 hours following infection when using anti-V serum. At 6 hours no staining was observed in two of the experiments. In one experiment, however, very weak perinuclear

Bovine embryo kidney cells 12 hrs. after infection with Asian influenza virus diluted 10^{-3}.

Fluorescent antibody technique using specific antisera and rhodamine-labelled rabbit anti-guinea-pig gamma globulin serum.

x 360.

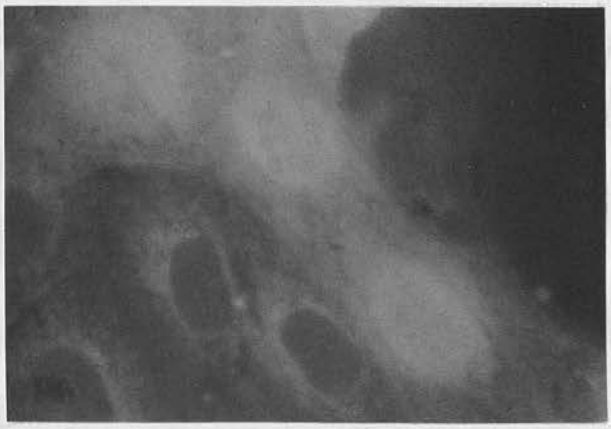

Figure 39.
Treated with guinea-pig anti-influenza serum.

Figure 40.
Treated with guinea-pig anti-S serum.

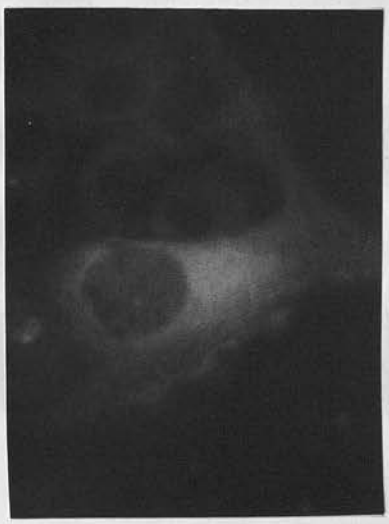

Figure 41.
Treated with guinea-pig anti-V serum.

fluorescence was noted in occasional cells. At 8 hours the cytoplasm showed diffuse fluorescence of moderate intensity with a definite perinuclear accumulation. The nuclei were generally unstained except for very rare cells which showed 1 - 4 well-defined nucleolus-like foci of low intensity. At 10 hours the picture was generally the same as at 8 hours, although more cells exhibited the described foci in the nucleus. At 12 hours the cytoplasm showed diffuse staining of moderate intensity. Perinuclear accumulation was still present, but, in addition, fluorescence also aggregated along the periphery of the cells (Figure 41). The described nuclear foci were present in many cells and were more intensely stained. At 24 hours the appearance of the fluorescing cells was essentially as at 12 hours.

The results of Experiment 46 may be summarised as follows (see Table 29):

Fluorescence due to S antigen was first observed 6 hours after infection as coarse granular staining of weak intensity which occupied the whole nucleus; by 8 hours S antigen could also be observed as weak perinuclear staining in the cytoplasm. In later preparations the nuclear staining became very bright, filling the whole nucleus, but the intensity of cytoplasmic staining remained quite low. V antigen became detectable 8 hours after infection as diffuse fluorescence of moderate intensity which accumulated in the perinuclear area; by 12 hours denser staining was observed along the periphery of the cells. The nuclei were generally unstained except for rare cells which showed well-defined nucleolus-like foci.

Titration results showed a constant rise in infective virus which was first measurable 8 hours after infection, i.e. when the S and V antigens were detectable by the fluorescent antibody technique. Haemagglutinins were not observed until 12 hours after infection.

Experiment 47.

The development of infectivity, haemagglutinating activity and specific fluorescence in bovine embryo kidney cells following inoculation with a large dose of an Asian strain of influenza virus.

The results described above were obtained from experiments in which dilute inocula containing approximately 10^6 EID_{50}/ml. were employed. In an attempt to induce an incomplete cycle of multiplication, parallel experiments employing inocula containing approximately 10^9 EID_{50}/ml. were carried out on the same day and under the same conditions as Experiment 46; in this way, ready comparison of titration results and stained preparations was made possible, and any differences which might occur with the fluorescent technique could thereby be detected.

The experimental conditions were exactly the same as those in Experiment 46 except that the cells were inoculated with 2 ml. of the virus suspension diluted 1 : 2 in Parker's medium; the multiplicity of infection possible was therefore approximately 10^3 EID_{50} units/cell. Titrations and fluorescent studies were carried out as before.

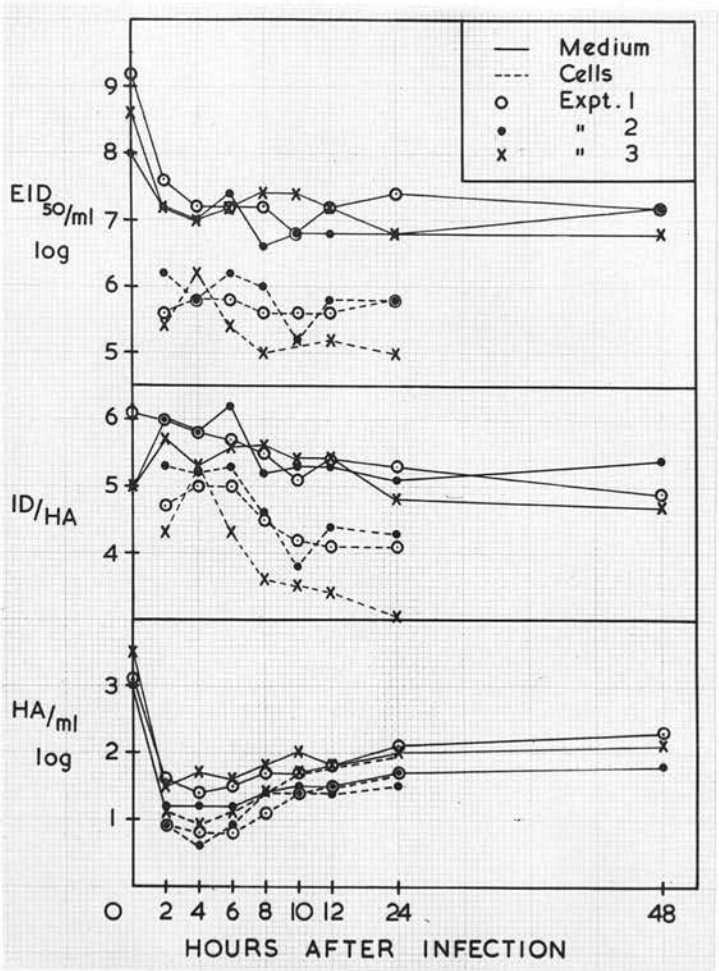

Figure 42.

The development of infectivity and haemagglutinating activity in bovine embryo kidney cells and their nutrient medium following inoculation with a large dose of an Asian strain of influenza virus.

Titration results. These are shown in Figure 42 and it may be seen that no increase in infectivity could be detected by the egg titration method. A significant rise in haemagglutinins was, however, apparent, being more striking in the cells than in the medium; this can presumably be explained by the presence in the medium of residual virus from the inoculum. On examining the ID/HA ratios it may be seen that during the

24 hours after infection a gradual decrease in ratio occurred in both medium and in cell suspensions.

TABLE 30. Comparison of the ID/HA ratios attained in the medium and in supersonic-treated cell suspensions under conditions of high and low multiplicity of infection (Experiments 46 and 47).

EXPERIMENT	ID/HA RATIOS AFTER 24 HOURS			
	Medium		Cell suspension	
	High	Low	High	Low
1	4.9	5.1	3.1	4.7
2	4.8	-	3.4	-
3	4.8	5.3	2.8	3.6

- = haemagglutinins not detected.

The ID/HA ratios achieved in these experiments were compared with those obtained at the same time intervals following infection with dilute inocula (Table 30) and it may be seen that although there is only a slightly lower ratio in the medium in the high inoculum experiments the ratio in the cells is considerably lower than in the dilute inoculum series. This may be an indication of incomplete virus formation.

Fluorescent antibody studies.

The development of specific fluorescence in the cells is shown in Table 31.

TABLE 31. The development of specific fluorescence in bovine embryo kidney cells infected with a large inoculum of Asian influenza virus.

HOURS AFTER INFECTION	ANTISERUM					
	WHOLE		ANTI-S		ANTI-V	
	CYTOPLASM	NUCLEUS	CYTOPLASM	NUCLEUS	CYTOPLASM	NUCLEUS
2	-	-	-	-	-	-
4	-	(+)	-	(+)	-	-
6	+	+	-	+	+	-
8	++	+	(+)	+	++	$+^{x}$
10	++	+	(+)	+	++	$++^{x}$
12	++	+	(+)	+(+)	++	$+^{x}$
24	++	+	(+)	+(+)	++	$+^{x}$

Intensity of fluorescence expressed by an arbitrary scale:

(+) = weak intensity; + = fair intensity;

++ = moderate intensity, etc.;

x = intensity in the described nuclear foci.

Whole antiserum. Specific fluorescence was first observed in the cells at 4 hours, when an occasional nucleus showing weak reticular staining could be seen. By 6 hours the majority of cells fluoresced; this fluorescence was primarily nuclear, occurring in a stippled pattern of coarse granules, but weak, diffuse, perinuclear cytoplasmic staining was also observed. After this time the cytoplasmic staining increased, becoming mainly diffuse throughout the cytoplasm, although accumulations in the perinuclear area were still apparent. By 10 hours peripheral accumulation was also observed in some cells and the number of cells showing such accumulations increased in later

Bovine embryo kidney cells after infection with Asian influenza virus (large and small inocula). Treated with guinea-pig anti-influenza serum and rhodamine labelled rabbit anti-guinea-pig gamma globulin serum.

x 360.

Figure 43.
8 hrs. after infection with virus diluted 10^{-3}.

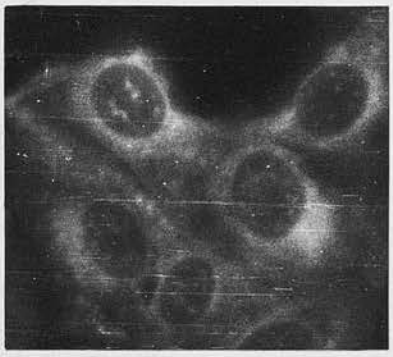

Figure 44.
8 hrs. after infection with virus diluted 1 : 2.

Figure 45.
10 hrs. after infection with virus diluted 10^{-3}.

Figure 46.
10 hrs. after infection with virus diluted 1 : 2.

Figure 47.
12 hrs. after infection with virus diluted 10^{-3},

Figure 48.
12 hrs. after infection with virus diluted 1 : 2.

Bovine embryo kidney cells after infection with Asian influenza virus (large and small inocula). Treated with guinea-pig anti-S serum and rhodamine labelled rabbit anti-guinea-pig gamma globulin serum.

x 360.

Figure 49.
8 hrs. after infection with virus diluted 10^{-3}.

Figure 50.
8 hrs. after infection with virus diluted 1 : 2.

Figure 51.
10 hrs. after infection with virus diluted 10^{-3}.

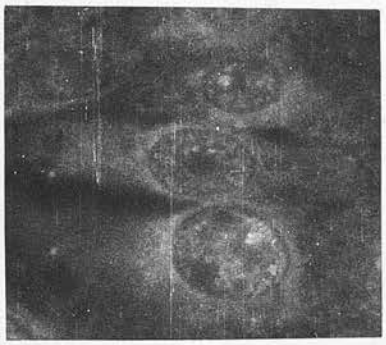

Figure 52.
10 hrs. after infection with virus diluted 1 : 2.

Figure 53.
12 hrs. after infection with virus diluted 10^{-3}.

Figure 54.
12 hrs. after infection with virus diluted 1 : 2.

preparations. In contrast to this, the intensity of nuclear staining showed only a very slight increase between 6 and 24 hours and was never seen to fill the nucleus as was the case in the experiments employing dilute inocula (Figures 43 - 48).

S antiserum. At 4 hours occasional cells could be seen showing a low degree of reticular fluorescence in the nucleus. This had increased by 6 hours and by 8 hours the majority of cells showed staining which was primarily nuclear; some cells also showed weak cytoplasmic fluorescence accumulated mainly in the perinuclear area. By 12 hours the intensity of the nuclear staining had increased slightly but was still considerably less than in the parallel cultures from the dilute inocula experiments (see Figures 49 - 54). The situation at 24 hours was similar; some cells showed only nuclear fluorescence, while in others, in addition to the nuclear staining, faint diffuse cytoplasmic fluorescence could also be seen. As with the whole antiserum the nuclear staining was never seen to fill the nucleus.

V antiserum. Although in one experiment weak cytoplasmic fluorescence was observed at 4 hours this was generally not detectable until 6 hours after infection when the staining was seen in approximately 50 per cent. of the cells as weak primarily perinuclear fluorescence. By 8 hours the intensity of staining had increased and it was possible to detect the presence of fluorescent foci in the nuclei of a few cells. The number of cells showing such foci increased in later preparations but no other staining could be seen in the nuclei. The

Bovine embryo kidney cells after infection with Asian influenza virus (large and small inocula). Treated with guinea-pig anti-V serum and rhodamine labelled rabbit anti-guinea-pig gamma globulin serum.

x 360.

Figure 55.
8 hrs. after infection with virus diluted 10^{-3}.

Figure 56.
8 hrs. after infection with virus diluted 1 : 2.

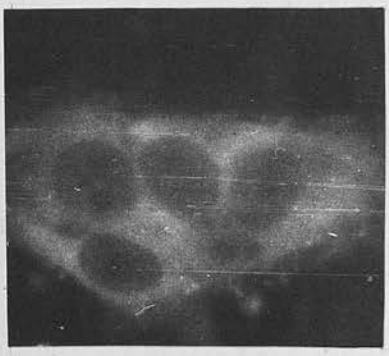

Figure 57.
10 hrs. after infection with virus diluted 10^{-3}.

Figure 58.
10 hrs. after infection with virus diluted 1 : 2.

Figure 59.
12 hrs. after infection with virus diluted 10^{-3}.

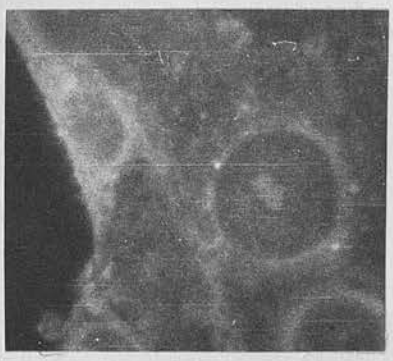

Figure 60.
12 hrs. after infection with virus diluted 1 : 2.

cytoplasmic staining remained in a diffuse pattern with peri-nuclear accumulation; from 10 hours peripheral concentration was also observed in some cells.(Figures 55 - 60).

When the observations made with the fluorescent antibody technique (Table 31) are compared with the results obtained from egg infectivity and haemagglutination titrations (Figure 42) the reduced amount of detectable S antigen might appear to be related to the lack of increase in infectious virus. The appearance of V antigen at 6 hours probably corresponds to the rise in haemagglutinin production which occurs mainly between 4 and 8 hours after infection.

The preparations described here were examined in parallel with those from the dilute inocula experiments (Experiment 46) and some differences were observed. Firstly, many more cells showed fluorescence which presumably reflected the higher multiplicity of infection employed. Secondly, although the sequence of appearance of both antigens was the same, they were detected earlier with large inocula. Thirdly, the nuclear fluorescence observed in cells treated with the whole and anti-S sera was never as great as that observed in similar preparations of cells infected with dilute inocula (Figures 43 - 54). These results indicate that a decreased production of S antigen per cell occurred when massive inocula were employed.

The growth of Asian influenza virus in HeLa cells.

Bovine embryo kidney cells are known to support the multiplication of "complete" influenza virus under normal conditions but HeLa cells have been found to support only the "incomplete" multiplication of the virus (Henle et al., 1955). This incomplete cycle of multiplication only occurs when the cells are exposed to high multiplicities of infection; with dilute inocula no demonstrable replication has been reported. However, the production of S and V antigens, measured by complement-fixation tests on disrupted infected cells, was found to follow the normal expected pattern of complete virus and no explanation could be given for the failure of production of infectious particles. It seemed of value, therefore, to follow by fluorescent antibody studies the development of specific antigens of a strain of influenza A virus in HeLa cells. Any elucidation of this true "incomplete" cycle might help to explain the findings obtained in Experiment 47, that a reduced amount of S antigen per cell was produced when bovine embryo kidney cells were inoculated with a large dose of virus.

Experiment 48.

The development of infectivity, haemagglutinating activity and specific fluorescence in HeLa cells following inoculation with an Asian strain of influenza virus.

Two series of experiments were carried out employing a high and a low multiplicity of infection and the experimental details were identical to those in the two previous experiments (46 and 47) except for the use of HeLa cell cultures instead of

bovine embryo kidney cells. Titrations and fluorescent studies were carried out as before.

TABLE 32. Haemagglutinin titrations and egg infectivity assays of medium and disrupted cells, following infection of HeLa cells with a dilute inoculum of Asian influenza virus.

HOURS AFTER INFECTION	VIRUS TITRES PER ML.LOG.			
	Medium		Cells*	
	EID_{50}	HA	EID_{50}	HA
0	7.0	3.7		
2	4.2	-	4.0	-
4	3.8	-	4.0	-
6	4.2	-	3.8	-
8	4.8	-	3.4	-
10	4.0	-	3.4	-
12	4.0	-	3.6	-
24	4.0	-	3.0	-
48	4.2	-		

* = after supersonic vibration.

Dilute inoculum.

Table 32 shows the titration results following infection with a dilute inoculum and it is readily seen that no rise in titre occurred throughout the interval investigated. Fluorescent microscopy of these cells revealed the development of no influenza antigens which could be detected by anti-S or anti-V serum or by antiserum to the whole virus, throughout the 24 hour period investigated.

Concentrated inoculum.

Titration results. The infectivity assays and haemagglutination titrations of medium and supersonically treated cells, following infection with concentrated inocula, are shown in Figure 61.

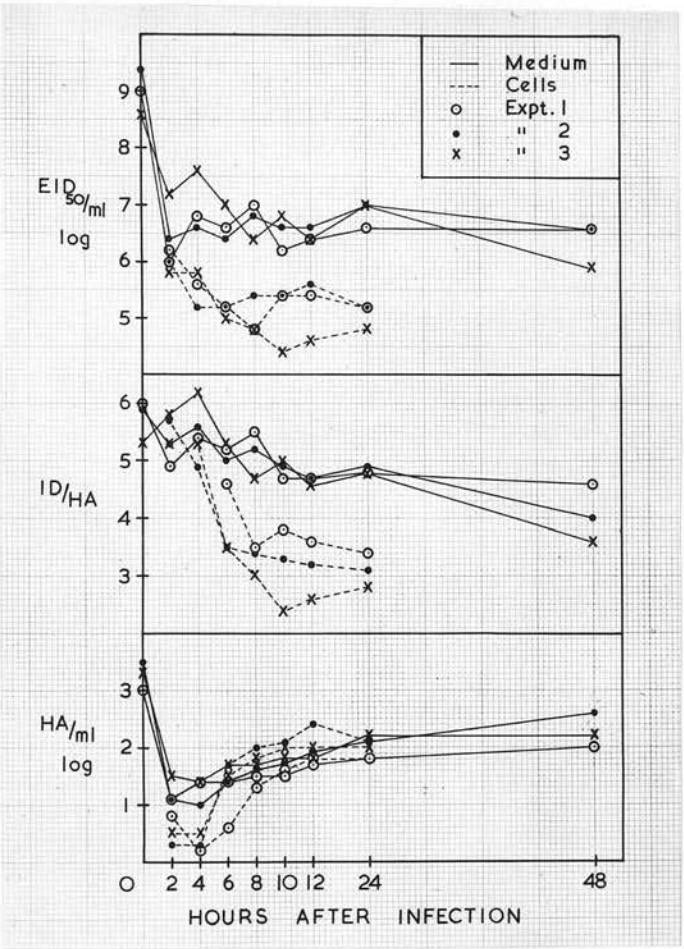

Figure 61.
The development of infectivity and haemagglutinating activity in HeLa cells and their nutrient medium following inoculation with a large dose of an Asian strain of influenza virus.

Haemagglutination titres first began to rise significantly between 6 and 8 hours after infection and rose steadily thereafter throughout the 24 hours examined. Infectivity titres showed no rise throughout; the values observed represent

residual seed virus. ID/HA ratios show a progressive decrease, indicating clearly the development of non-infectious haemagglutinins, more apparent in the intracellular phase, i.e. in the supersonically disrupted cells. It must be borne in mind, however, that intracellular haemagglutinins were also encountered in the intracellular phase with bovine embryo kidney cells.

Fluorescent antibody studies. The appearances of the preparations stained with anti-S, anti-V and whole antiserum in the fluorescent microscope are summarised in Table 33.

TABLE 33. The development of specific fluorescence in HeLa cells infected with a large inoculum of Asian influenza virus.

HOURS AFTER INFECTION	ANTISERUM					
	Whole		Anti-S		Anti-V	
	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus
2	-	-	-	-	-	-
4	-	-	-	-	-	-
6	(+)	-	-	(+)	(+)	-
8	-	(+)	-	+	+	-
10	++	+	-	+	++	$(+)^x$
12	+++	+	(+)*	+	+++	$+^x$
24	+++	+	(+)*	+	+++	$+^x$

Intensity expressed by an arbitrary scale:

(+) = weak intensity; + = fair intensity;

++ = moderate intensity, etc.;

* = the fluorescence recorded occurred in only a very few of the cells showing nuclear fluorescence;

x = intensity in the described nuclear foci.

Whole antiserum. Staining in whole antiserum-treated cells was not detected until 8 hours, when the nuclei revealed coarse, granular fluorescence of weak intensity and sparse distribution. By 10 hours the nuclear staining had increased slightly and diffuse cytoplasmic staining of moderate intensity was observed. The intensity of the cytoplasmic fluorescence continued to increase, whereas the nuclei remained weakly stained (Figure 62).

S antigen first appeared in the nucleus from 4 to 6 hours after infection. Its pattern, throughout the time period observed, remained one of scattered distribution of weakly fluorescing coarse granules (Figure 63). The nucleus never appeared thoroughly filled with antigen as was the case with beef embryo kidney cells exposed to a low multiplicity of infection. Its intensity increased somewhat by 8 hours but did not thereafter further increase; this was in contrast to the development of brilliantly intense S antigen in the case of "complete" virus formation. In addition, the S antigen remained largely confined to the nucleus. Occasional cells at 12 and 24 hours were seen to exhibit scattered cytoplasmic granules, but these represented only a very small percentage of the total number of fluorescing cells.

V antigen, on the other hand, developed primarily in the cytoplasm. It made its appearance 6 hours after infection and gradually increased up to 12 hours, remaining intensely fluorescent at 24 hours (Figure 64). In all preparations the

HeLa cells 12 hrs. after infection with Asian influenza virus diluted 1 : 2. Treated with specific antisera and rhodamine-labelled rabbit anti-guinea-pig gamma globulin serum.

x 360.

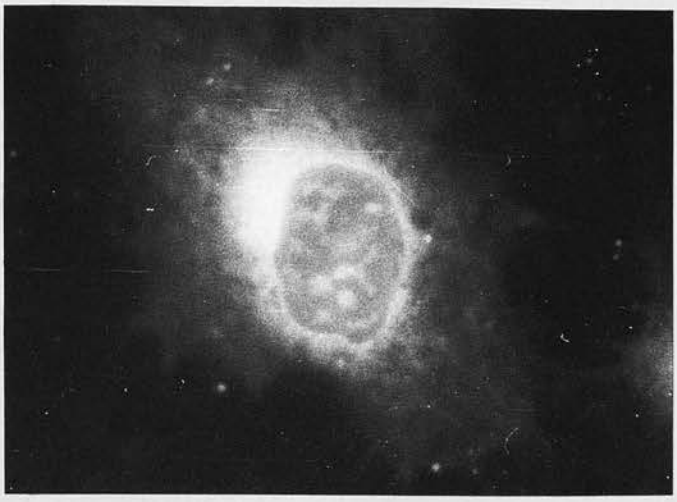

Figure 62.

Treated with guinea-pig anti-influenza serum.

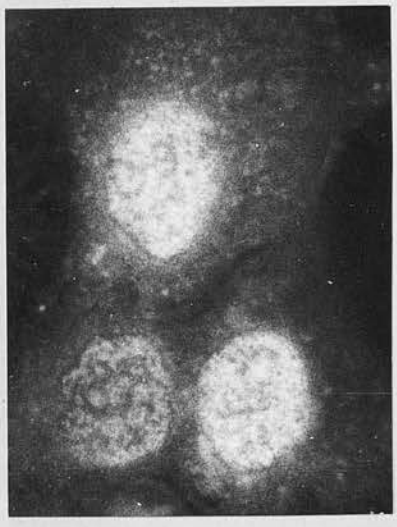

Figure 63.

Treated with guinea-pig anti-S serum.

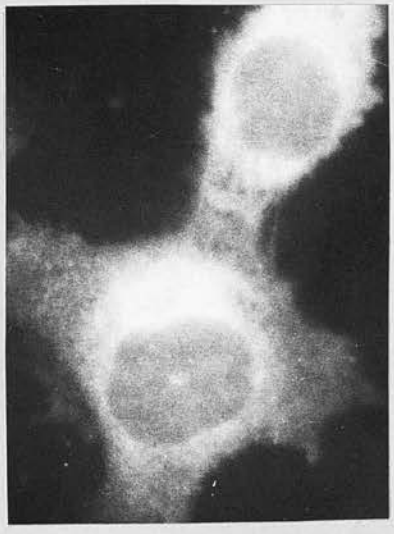

Figure 64.

Treated with guinea-pig anti-V serum.

V antigen appeared to remain concentrated perinuclearly in contrast to the peripheral localisation noted in the case of "complete" virus formation. Foci of V antigen began to appear in the nucleus at 10 hours and increased somewhat at 12 and 24 hours. These foci, which were definite rounded bodies with a striking resemblance to nucleoli, were similar in appearance to those seen in later stages of infection of beef embryo kidney cells, at both high and low multiplicities of infection.

The results obtained can be seen to resemble those achieved in Experiment 47, when bovine embryo kidney cells were infected with a large dose of influenza virus. The only striking difference noted was that the vast majority of infected HeLa cells failed to exhibit the presence of any S antigen in the cytoplasm detectable by the fluorescent antibody techniques employed. This finding which will be discussed later might be related to the inability of HeLa cells to support a complete cycle of multiplication.

TABLES

TABLE 1. The development of cytopathic changes in HeLa cells after inoculation with the Herts, B1 and Cal strains of Newcastle disease virus.

STRAIN	DILUTION	C P E			
		Days after inoculation			
		1	2	3	4
HERTS	-6	+	+++	++++	++++
	-7	-	+	+++	++++
	-8	-	±	+++	++++
	-9	-	-	-	-
	-10	-	-	-	-
B_1	0	++	++++	++++	++++
	-1	-	+++	++++	++++
	-2	-	++	++++	++++
	-3	-	+	+++	++++
	-4	-	-	++	++
CAL	-1	++	++++	++++	++++
	-2	++++	++++	++++	++++
	-3	+++	++++	++++	++++
	-4	+++	++++	++++	++++
	-5	+	+++	++++	++++

TABLE 2. The correlation between development of cytopathic effect and haemagglutinins in HeLa cells infected with NDV.

INOCULUM	DAYS AFTER INOCULATION												
	C P E						H A						
	1	2	3	4	5	7	Inoc.	1	2	3	4	5	7
-1	++ ++ ++	+++ +++ +++	+++ +++ +++	+++ +++ +++	++	++	++++	-	++	++	-	-	-
-2	+ + ++	++ ++ ++	+++ +++ +++	+++ +++ +++	++	++	++++	-	++	++	-	-	-
-3	- - ±	++ + ++	+++ +++ +++	+++ +++ +++	++	++	++	-	± -	++++	-	-	-
-4	- - -	+ ++ ++	+++ +++ +++	+++ +++ +++	+++	+++	++	-	-	++++	+++	-	-
-5	- - -	+ + +	+++ +++ +++	+++ +++ +++	+++	+++	++	-	-	++++	++++	+	-
-6	- - -	+ + +	+++ +++ +++	+++ +++ +++	++	+++	+	-	-	++++	++++	+	+
-7	- - -	- + +	+++ +++ +++	+++ +++ +++	+++	+++	+	-	-	++++	++++	+	+
-8	- - -	+ + +	+++ +++ +++	+++ +++ +++	+++	+++	+	-	-	+++	++++	++	+
-9	- - -	- - -	- ++ +++	± +++ +++	+++	+++	+	-	-	++	++++	+++	++++

TABLE 3. The effect of dilution on the infectivity, haemagglutinating activity and CPE of NDV in HeLa cells.

DILUTION	DAYS	CELL COUNTS	CPE	VIRUS TITRES PER ML. LOG					
				Fluid phase			Cellular phase		
				CPD_{50}	HA	CPD/HA	CPD_{50}	HA	CPD/HA
UD	1	7.5×10^3	++++	6.5	1.5	5.0	0.3	-	-
	2	6.9	++++	5.8	1.8	4.0	-	-	-
	3	1.9	++++	6.3	1.8	4.5	-	-	-
	4	1.3	++++	5.8	2.1	3.7	-	-	-
	5	2.5	++++	3.6	2.1	1.5	-	-	-
-2	1	20×10^3	++++	5.8	0.6	5.2	-	-	-
	2	5	++++	6.8	1.2	5.6	-	-	-
	3	2.5	++++	5.5	1.2	4.3	-	-	-
	4	2.5	++++	5.3	1.2	4.1	-	-	-
	5	3.3	++++	5.5	1.2	4.3	-	-	-
-4	1	45×10^3	+++	5.3	-	-	3.5	0.6	2.9
	2	12	++++	6.3	0.9	5.4	1.3	-	-
	3	5.0	++++	4.8	1.2	3.6	-	-	-
	4	6.3	++++	5.8	1.5	4.3	-	-	-
	5	3.3	++++	4.8	1.5	3.3	-	-	-
-6	1	305×10^3	+	2.5	-	-	0.3	-	-
	2	170×10^3	+++	5.5	0.9	4.6	3.5	1.5	2.0
	3	18.0x	++++	6.8	1.8	5.0	-	-	-
	4	5.0	++++	6.3	1.5	4.8	-	-	-
	5	6.3	++++	6.8	1.8	5.0	-	-	-
-8	1	485×10^3	-	1.3	-	-	-	-	-
	2	660	+	2.8	-	-	2.0	-	-
	3	295	++	5.5	1.2	4.3	1.5	0.6	0.9
	4	80	+++	6.0	2.1	3.9	0.5	-	-
	5	55	++++	6.0	2.1	3.9	1.5	0.6	0.9
Control	1	370×10^3	-						
	2	710	-						
	3	770	-						
	4	1080	-						
	5	1000	-						

TABLE 6. The survival of HeLa cells after inoculation with varying doses of NDV.

DILUTION	TUBE	DAYS AFTER INOCULATION			TITRE AFTER 3 DAYS	DAYS AFTER REMOVAL OF VIRUS.					
		1	2	3		3	4	5	6	7	10
10^{-2}	1	+++	++++	++++	8.7	-	±	++	+	±	++
	2	+++	++++	++++		-	±	+	+	++	+
	3	+++	++++	++++		±	±	+++	++	+	-
	4	+++	++++	++++		-	±	+	+	++	++
	5	+++	++++	++++		-	±	+	+	+	+
10^{-3}	1	+++	++++	++++	10.5	±	+	++	+++	++	+
	2	+++	++++	++++		±	+	++	+++	+	+
	3	+++	++++	++++		±	+	+++	+++	++	++
	4	+++	++++	++++		+	+	+	+	+	-
	5	+++	++++	++++		±	+	+++	+++	++	-
10^{-4}	1	++	+++	++++	11.3	+	++	+++	+++	+	-
	2	++	+++	++++		+	++	++	+++	+	-
	3	++	+++	++++		+	++	+++	+++	+	-
	4	++	+++	++++		+	++	++	++	+	-
	5	++	+++	++++		++	++	+	+	+	+
10^{-5}	1	+	+++	++++	11.2	+	++	++	++	+	-
	2	+	+++	++++		+	++	++	++	+	+
	3	+	+++	++++		+	++	++	+	±	+
	4	+	+++	++++		+	++	+++	++	±	+
	5	+	+++	++++		+	++	++	+	±	+

+ = CPE

+ = growth of cells

TABLE 8. The growth of HeLa cells after previous treatment with NDV.

INOCULUM	DAYS AFTER INOCULATION													
	C P E					H A					CELL COUNT x 10^4			
	1	2	4	5	6	Before inoc.	2	4	5	6	2	4	5	6
Hanks' 4° C.	-	-	-	-	-	-	-	-	-	-	8.0	15.2	26.0	6.0
Hanks' 20° C.	-	-	-	-	-	-	-	-	-	-	4.9	8.1	10.0	16.0
NDV 10^{-4} 4° C.	-	+	+++	++++	++++	-	+	+	-	-	1.6	0.9	1.8	1.8
NDV 10^{-4} 20° C.	-	-	+++	++++	++++	-	+	+	-	-	2.0	1.4	1.6	1.1
NDV 10^{-1} 4° C.	++++	++++	++++	++++	++++	+	+++	-	-	-	0.4	0.7	1.4	0.7
NDV 10^{-1} 20° C.	++++	++++	++++	++++	++++	-	+++	-	-	-	0.1	0.5	0.7	0.4

TABLE 9. Neutralisation of the CPE of NDV in HeLa cells by homologous antiserum.

INOCULUM		DAYS AFTER INOCULATION							
		C P E				H A			
Virus	Antiserum	1	2	3	4	1	2	3	4
Nil	Nil	- - -	- - -	- - -	- - -	-	-	-	-
10^{-2}	Nil	+++ +++ +++	++++ +++ +++	++++ ++++ ++++	++++ ++++ ++++	++++	++++	-	-
Nil	1/500	- - -	- - -	- - -	- - -	-	-	-	-
10^{-2}	1/10	- - -	- - -	- - -	- - -	-	-	-	-
10^{-2}	1/100	- - -	- - -	+ + +	+++ +++ +++	-	-	-	++++
10^{-2}	1/250	± - -	+ + +	+++ +++ +++	++++ ++++ ++++	-	-	++++	++++
10^{-2}	1/500	+ + +	+++ +++ +++	++++ ++++ ++++	++++ ++++ ++++	-	++	++++	++++
10^{-2}	1/1000	++ ++ ++	++++ ++++ ++++	++++ ++++ ++++	++++ ++++ ++++	-	++++	-	-

TABLE 10. Neutralisation of the CPE of NDV in HeLa cells by antiserum added after infection with the virus (Experiment 13)

INOCULUM		DAYS AFTER INOCULATION				
		CPE			HA	
Virus	Antiserum	1	2	3	0	3
10^{-6}	Nil	+ + +	+++ +++ +++	++++ ++++ ++++	++	+++
10^{-6}	10^{-2}	± ± ±	± ± ±	± + +		+
10^{-6}	10^{-3}	± ± ±	++ ++ ++	+++ +++ +++		++
10^{-6}	10^{-4}	+ + +	+++ +++ +++	+++ +++ +++		++
10^{-6}	10^{-5}	+ + +	++++ ++++ ++++	++++ ++++ ++++		+++
10^{-9}	Nil	± - -	++ ++ ++	+++ +++ +++	-	+++
10^{-9}	10^{-2}	- - -	- - -	- - -		-
10^{-9}	10^{-3}	- - -	- ± ±	- + +		-
10^{-9}	10^{-4}	- - -	+ + +	++ ++ ++		+++
10^{-9}	10^{-5}	- - -	++ ++ -	+++ +++ -		+++

TABLE 11. Neutralisation of the CPE of NDV in HeLa cells by antiserum added after infection with the virus (Experiment 14)

INOCULUM		DAYS AFTER INOCULATION							
		Expt. 1. C P E				Expt. 2. C P E			
Virus	Antiserum	1	4	6	8	1	4	6	8
10^{-3}	Nil	++ ++ ++	+++ +++ +++	++++ ++++ ++++	++++ ++++ ++++	++ ++ ++	+++ +++ +++	++++ ++++ ++++	++++ ++++ ++++
10^{-3}	10^{-1}	+ + +	+++ +++ +++	+++ +++ +++	+++ +++ +++	- - -	- - -	++ ++ ++	++++ ++++ ++++
10^{-3}	10^{-2}	++ ++ ++	+++ +++ +++	++++ ++++ ++++	++++ ++++ ++++	- + -	++ ++ ++	++++ ++++ ++++	++++ ++++ ++++
10^{-7}	Nil	- - -	++ ++ ++	++++ ++++ ++++	++++ ++++ ++++	- - -	- - +	++ ++ +	++++ ++++ ++++
10^{-7}	10^{-1}	- - -	- - -	- - -	++ ++ ++	- - -	- - -	- - -	- + -
10^{-7}	10^{-2}	- - -	- - -	++ - +	+++ ++ +++	- - -	- - -	- - -	+ ++ ++

TABLE 12. The CPE and growth of NDV in HeLa cells treated with 0.01 M sodium periodate.

TREATMENT OF CELLS		CPE Days after inoculation			TITRE AFTER 3 DAYS	
$NaIO_4$	Virus	1	2	3	TCD_{50}	HA
0.01 M	Nil	- - - -	- - - -	- - - -	-	-
	10^{-1}	- - - -	- - - -	- - - -	-	-
	10^{-6}	- - - -	- - - -	- - - -	-	-
Nil	Nil	- - - -	- - - -	- - - -	-	-
	10^{-1}	++++ ++++ ++++ ++++	++++ ++++ ++++ ++++	++++ ++++ ++++ ++++	1.0	1.5
	10^{-6}	+ + + +	+++ +++ +++ +++	++++ ++++ ++++ ++++	4.8	1.5

TABLE 13. The CPE of NDV in HeLa cells treated with 0.01 M, 0.001 M and 0.0001 M sodium metaperiodate.

TREATMENT OF CELLS		CPE Days after inoculation		
$NaIO_4$	Virus	1	2	3
0.01 M	Nil	- - -	- - -	- - -
	10^{-1}	- - -	- - -	- - -
	10^{-6}	- - -	- - -	- - -
0.001 M	Nil	- - -	- - -	- - -
	10^{-1}	+++ +++ +++	++++ ++++ ++++	++++ ++++ ++++
	10^{-6}	+ + +	++ +++ ++	+++ +++ +++
0.0001 M	Nil	- - -	- - -	- - -
	10^{-1}	+++ +++ +++	++++ ++++ ++++	++++ ++++ ++++
	10^{-6}	+ + +	+++ +++ +++	+++ +++ +++
Nil	Nil	- - -	- - -	- - -
	10^{-1}	+++ +++ +++	++++ ++++ ++++	++++ ++++ ++++
	10^{-6}	+ + +	++ +++ ++	+++ +++ +++

TABLE 14. The CPE and growth of NDV in HeLa cells treated with dilutions of 0.01 M sodium metaperiodate.

TREATMENT OF CELLS		C P E Days after inoc^{n}		HA TITRE AFTER
$NaIO_4$	Virus	1	3	3 DAYS
0.01 M undiluted	Nil	- -	- -	-
	10^{-1}	- -	- -	0.6
	10^{-6}	- -	- -	-
1/2	Nil	- -	- -	-
	10^{-1}	- -	- -	0.6
	10^{-6}	- -	- -	-
1/4	Nil	- -	- -	-
	10^{-1}	+++ +++	+++ +++	0.6
	10^{-6}	- -	- -	-
1/8	Nil	- -	- -	-
	10^{-1}	+++ +++	+++ +++	1.2
	10^{-6}	+ ++	+++ +++	1.2
1/10	Nil	- -	- -	-
	10^{-1}	+++ +++	+++ +++	1.2
	10^{-6}	+ +	+++ +++	1.2
Nil	Nil	- -	- -	-
	10^{-1}	+++ +++	+++ +++	1.2
	10^{-6}	+ +	+++ +++	1.2

TABLE 16. The effect of serial passage of diluted PR8 virus in monkey kidney cultures on its infectivity for embryonated eggs and monkey kidney cultures.

PASSAGE NUMBER	LOG VIRUS TITRES PER ML. FLUID AFTER 72 HOURS					
	HA	EID_{50}	TCD_{50}	EID/HA	TCD/HA	EID/TCD
1	1.8	+	+	-	-	-
2	2.6	+	5.6	-	3.0	-
3	2.6	+	7.2	-	4.6	-
4	2.6	+	6.2	-	3.6	-
5	2.4	+	6.2	-	3.8	-
6	2.7	8.9	5.6	6.2	2.9	3.3
7	2.4	8.5	6.9	6.1	4.5	1.6
8	2.9	8.2	6.5	5.3	3.6	1.7
9	2.7	7.9	6.9	5.2	4.2	1.0
10	2.6	7.5	6.2	4.9	3.6	1.3
11	2.0	7.9	6.5	5.9	4.5	1.4
12	2.1	8.2	6.5	6.1	4.4	1.7
13	2.7	8.2	6.5	5.5	3.8	1.7
14	2.7	8.2	7.2	5.5	4.5	1.0
15	2.4	8.2	6.5	5.8	4.1	1.7
16	2.4	8.8	6.9	6.4	4.5	1.9
17	2.9	8.6	6.2	5.7	3.3	2.4
18	2.6	9.2	6.5	6.6	3.9	2.7
19	2.6	8.4	6.9	5.8	4.3	1.5
20	2.9	8.4	6.2	5.5	3.3	2.2
21	2.3	8.2	6.9	5.9	4.6	1.3
Mean values 6 - 21	2.5	8.7	6.6	6.2	4.1	2.1

+ = not titrated.

TABLE 17. The effect of serial passage of undiluted PR8 virus in monkey kidney cultures on its egg infectivity and haemagglutinating activity.

PASSAGE NUMBER	LOG.VIRUS TITRES PER ML.FLUID AFTER 24 HRS.					
	Washed cultures A			Unwashed cultures B		
	EID_{50}	HA	ID/HA	EID_{50}	HA	ID/HA
1	7.6	2.4	5.2	8.2	2.9	5.3
2	6.8	0.9	5.9	7.4	2.2	5.2
3	7.8	1.2	6.6	7.2	1.5	5.7
4	8.2	1.7	6.5	7.2	1.2	6.0
5	8.4	1.7	6.7	8.4	1.8	6.6
6	7.8	1.9	5.9	7.8	2.1	5.7
7	7.4	1.1	6.3	6.2	1.3	4.9
8	6.6	-	-	6.4	0.7	5.7
9	7.8	1.4	6.4	7.0	1.2	5.8
10	8.0	1.9	6.1	5.8	1.5	4.3
11	6.6	0.9	5.7	5.8	0.7	5.1
12	7.0	0.8	6.2	6.4	0.8	5.6
13	8.0	1.4	6.6	7.2	1.7	5.5
14	6.2	1.1	5.1	5.4	1.3	4.1
15	6.0	1.2	4.8	5.4	-	-
16	7.2	1.5	5.7	7.0	1.4	5.6
17	7.2	1.4	5.8	6.8	1.7	5.1
Mean values	7.4	1.4	6.0	6.9	1.3	5.6

TABLE 28. Development of infectivity, haemagglutinating activity, specific fluorescence and nucleic acid changes in bovine embryo kidney cells following inoculation with large and small doses of standard PR8 virus and EUP 2 PR8 virus.

INOCULUM	HRS.	VIRUS TITRES PER ML. LOG.						FLUORESCENCE WITH ANTI-S SERUM		TYPICAL CHANGES
		Medium			Cells*					
		EID_{50}	HA	ID/HA	EID_{50}	HA	ID/HA	Nucleus	Cyto-plasm	AO
PR8 1 : 2	2	7.0	1.4	5.6	5.8	0.8	5.0			
	12	7.2	2.3	4.9	6.0	2.3	3.7	+	±	+
	24	8.2	2.6	5.6	6.2	2.7	3.5	+	+	+
	48	7.0	2.9	4.1	x			++	++	+
PR8 1 : 1000	2	4.6	-	-	4.4	-	-			
	12	7.4	1.7	5.7	6.2	1.8	4.4	+++	+	-
	24	7.4	1.9	5.5	6.4	2.1	4.3	+++	+	-
	48	7.0	2.3	4.7	x			+++	++	-
PR8 UP 2 1 : 2	2	5.2	1.4	3.8	4.8	0.7	4.1			
	12	5.4	1.6	3.8	3.8	2.0	1.8	++	-	+
	24	5.8	2.1	3.7	4.6	2.3	2.3	+++	+	+
	48	5.8	2.5	3.3	x			++	(+)	+

AO stained with acridine orange
* after supersonic vibration
x not titrated

DISCUSSION

DISCUSSION

Evidence that Newcastle disease virus (NDV) and influenza virus differ in certain growth characteristics has already been reported by other workers. In the present study the cytopathic effect of these viruses was examined in various tissue cultures and the growth of the viruses, including the possible formation of incomplete virus, was investigated. Several differences were observed.

The cytopathic effect of Newcastle disease virus and influenza virus.

The cytopathic effect associated with NDV infection in HeLa cells could conveniently be described in three phases; these included the appearance of circular foci of infection termed "microplaques", the ensuing formation of syncytial masses followed by complete degeneration of the cell sheet. The changes were observed with both virulent and avirulent strains, although they occurred more slowly with the avirulent strain, which is in agreement with results obtained by Bang and Warwick (1957). Syncytia or giant cells have already been reported as being associated with NDV infection in monkey kidney cells (Chanock, 1955) and the cytopathic effect can thus be classified in Enders' group 3 (Enders, 1954).

Other viral agents inducing similar changes in tissue culture include measles virus (Enders and Peebles, 1954), mumps virus (Henle et al., 1954), parainfluenza II (Chanock, 1956) and rinderpest virus (Plowright and Ferris, 1957). The changes

induced in HeLa cells by mumps virus were described by Henle et al. (1954) as "cytolytic" and these authors suggested that the effect was related to the haemolytic property of the virus. Newcastle disease virus likewise possesses a haemolysin and the occurrence of a cytopathic effect resembling that produced by mumps virus was therefore feasible.

Influenza virus which is not haemolytic has been found to induce a different cytopathic effect and so far no evidence has come to light, either in the literature or in the present study, for the formation of syncytia in influenza-infected cells. Apart from the cytoplasmic changes such as granulation and vacuolation described by other workers (Mogabgab et al., 1955; Negroni and Tyrrell, 1959) it was possible in the present investigation to detect nuclear changes in cells infected with the PR8 strain of influenza A; these changes were seen in preparations stained with either haematoxylin and eosin or with acridine orange and gave the impression of intranuclear vacuoles separated by strands of darker staining material. These vacuoles may be analogous to the "peculiar clear areas" observed by Morgan and Rose (1959) in electronmicrographs of influenza-infected cells. These workers suggested that the nuclear alterations seen in such cells were related to the replication of the soluble antigen of influenza which is known to multiply within the nucleus. The finding in the present investigation that different strains of influenza A virus, which are known to have a different nucleic acid composition (Ada and Perry, 1956),

induce different nuclear changes, provides support for this suggestion.

Certain results were obtained in the present study which indicated that the nuclear changes detected by the acridine orange technique were not related to the presence of the S antigen itself but rather to some continuation of the infectious process in which RNA was produced in large amounts but was not completed to form S antigen. Further support for this interpretation is provided by the finding that S antigen makes up only 14 per cent. of the total infective particle (Frisch-Niggemeyer and Hoyle, 1956) which might not be detectable by the acridine orange staining method.

Correlation of cytopathic effect with virus multiplication.

An attempt has been made to correlate the appearance and extent of cytopathic changes with multiplication of the virus. When dilute inocula of influenza or NDV were employed, the development of cytopathic changes was usually accompanied closely by an increase in infectious and haemagglutinating virus; the only exception was with influenza in HeLa cells, where no rise in infectious virus was observed, although an increase in haemagglutinins did occur. This was in agreement with the findings of Henle et al. (1955), who observed an incomplete cycle of multiplication in this system. With large inocula, on the other hand, an increase in infective virus could not be detected in any of the systems examined, but cytopathic changes did occur

concomitantly with the production of haemagglutinins.

It was noted in the case of NDV that the number of cells surviving infection was greater following a large dose of virus. When HeLa cells, which had survived infection with NDV, were subcultured they were found to be susceptible to further infection with the same virus. It has been shown by Ciecura et al. (1957) that HeLa cells surviving NDV infection are more resistant to further infection than the original cells. Possibly the cells used in the present study did not receive sufficient subculture.

The addition of antiserum to cells following infection with NDV partially inhibited the development of cytopathic changes. This indicated that the virus was prevented from spreading through the medium and suggests the occurrence of a situation similar to that observed with herpes B in monkey kidney or HeLa cells (Black and Melnick, 1955) and herpes zoster and chickenpox in human amnion cells (Taylor-Robinson, 1958). It is also conceivable that a persistent infection may occur in the presence of immune serum similar to that observed with polio virus (Ackermann and Kurtz, 1955).

When the cell receptors were destroyed by means of sodium metaperiodate previous to infection with NDV, cytopathic changes and virus multiplication were inhibited. It was later found that the virus particle itself was also affected by this treatment; this was in agreement with findings obtained with influenza virus (Liu et al., 1956). Nevertheless, it seems legitimate to

suggest that adsorption of the virus was prevented to a certain extent by alteration of the cell receptors, thereby preventing virus multiplication.

Evidence for a toxic effect was obtained when NDV was added to HeLa cells before culturing, the number of cells surviving being inversely proportional to the size of the inoculum. This may be related to the cytotoxic effect first examined by Moore and Diamond (1953) and later by Prince and Ginsberg (1957 a, b) and by Eaton et al. (1960). A toxic reaction in the rabbit eye without increase in infectious virus has recently been reported (Oh and Evans, 1960). In animal experiments, Davenport (1952) found that after inoculation of massive doses of Newcastle disease virus into mouse lung, cellular injury occurred without multiplication of infective virus. No evidence for production of haemagglutinins was obtained and addition of antiserum following the virus did not diminish the toxic effect.

Cells infected with large doses of influenza A virus did not survive infection to the same extent as those infected with NDV. A toxic effect was thought to be associated with influenza infection of tissue culture cells, as more damage was produced by a large than by a small inoculum. Further experiments furnished support for this observation and the extent of the cytopathic changes produced appeared to be related to the amount of virus present in the inoculum. With allantoic fluid which had undergone two undiluted passages and thus had a lowered

infectivity titre, the cellular damage was considerably less than that observed with a standard virus preparation. This was not unexpected as incomplete virus preparations have been shown to be less toxic than complete preparations (Bernkopf, 1950; McKee, 1951; Manire, 1957). In virus preparations which had been concentrated by red cell adsorption complete destruction was observed with little increase in infectious virus.

It has been suggested that the production of a cytopathic effect by the virus in HeLa cells without multiplication of infective virus is due to a toxic effect (Henle et al., 1955). It is possible, however, that there is more than one factor responsible for the cellular alterations induced in tissue culture cells by influenza virus. This was found to be the case when adenovirus type 5 was grown in HeLa cells (Pereira, 1958). The non-toxic factor may involve some alteration to the cellular metabolism due to the lack of a key nutrient. In this connection Sanders (1957) observed that only when the virus productive capacity is exhausted do cytopathic changes become apparent.

Production of incomplete virus.

No concrete evidence was obtained for the formation of incomplete virus by Newcastle disease virus in HeLa cells. The lack of increase of infectious virus when large inocula were employed suggested that an incomplete cycle of multiplication was occurring but the results could not be considered conclusive, as considerable heat inactivation was involved. Although the occurrence of an incomplete cycle in Newcastle disease virus infections was originally indicated by the work of Granoff et

al. (1950) and of Nadel and Eisenstark (1955), further experiments did not provide evidence for this (Granoff, 1955 b).

The results obtained with large inocula of influenza virus in monkey kidney cells did, on the other hand, provide some evidence of incomplete virus formation although this was never so striking as the effect encountered in eggs (von Magnus, 1951 b) and in de-embryonated eggs (Bernkopf, 1950). When the PR8 strain of influenza virus was passed undiluted in monkey kidney cultures to give an initial multiplicity of infection of 10^3 EID_{50} per cell the ID/HA ratios were of the order of 10^6 and were similar to those obtained in serial passage of diluted material. However, on increasing the virus-cell ratio to 10^4 EID_{50} per cell a decrease in the ID/HA ratio was observed following undiluted serial passage. It was not possible in the present study to measure the amount of virus actually taking part in the infection when incomplete virus was produced, but it seems likely that multiple infection was possible. Cairns and Edney (1952) obtained results indicating that a high multiplicity of infection was not necessary for incomplete virus formation but a more recent review (Liu et al., 1956) indicates that multiplicity of infection is an important factor.

It was also possible to demonstrate non-infectious haemagglutinins in monkey kidney cells when undiluted egg passaged fluids were used as seed. The phenomenon was most pronounced when material from the second undiluted egg passage (UP 2) was used. Dilution of this seed resulted in the formation of fully infectious virus, whereas concentration of the

seed resulted in the production of a larger amount of non-infectious haemagglutinins. The phenomenon appears, therefore, to be closely related to the virus particle and is thus similar to that observed in eggs.

In experiments with virus partially inactivated by incubation at 37° C. there was no evidence of incomplete virus production in spite of the fact that these seeds had ID/HA ratios comparable to those of undiluted egg passage fluids. These results seem compatible with the findings of Paucker and Henle (1955), who observed that, with similar infectivity and haemagglutinating titres, yields from undiluted egg passage fluids contained 100 times more incomplete virus than yields from heated standard virus. It is more difficult to explain our data in the light of the findings of Horsfall (1954) who carried out experiments with mixtures of non-infective and infective particles, and reported that non-infective particles did cause definite reduction in the number of infective particles found in the yield. The fact that these results were obtained in tissue cultures, must, however, be borne in mind when comparing them with findings obtained in other host cell systems.

The original observation by Ada and Perry (1956) that virus preparations of low infectivity have a decreased RNA content may provide an explanation of the differences between undiluted egg passage and heat inactivated virus. The egg passage virus is actually deficient in nucleoprotein due to an upset in the metabolism of the host cell, whereas the heat inactivated virus contains the same amount of nucleic acid as

standard virus although part of this has been denatured by heating; this has been confirmed in an electron microscope study in which the two types of virus were found to differ morphologically (Birch-Andersen and Paucker, 1959). In my experiments employing the acridine orange staining technique it was not possible to detect differences in the nucleic acid distribution in cells producing complete virus and in cells producing incomplete virus. Strain differences could, however, be detected by this method which is consistent with the findings of Ada and Perry (1956) that strains of influenza virus differ in their nucleic acid content.

A possible explanation of the less striking production of incomplete virus in monkey kidney tissue culture encountered in the present study may be provided by failure to adapt the virus to this system. On the other hand, this suggestion is not compatible with the findings of Heath and Tyrrell (1958) who succeeded in adapting the PR8 strain of influenza virus to growth in monkey kidney cells; the titre in mice and in monkey kidney cultures was about 5000 fold higher relative to the titre in eggs than it had been before passage, but it was never as high as the egg titres encountered at the start of an undiluted passage series. In the present study the virus showed no significant alteration in titres throughout 21 passages in monkey kidney tissue culture. It is conceivable that the titre realised in tissue culture, which was approximately 10^2 lower than that attained in eggs, may not have been great enough to initiate the production of incomplete virus.

It is interesting to speculate why higher titres consistently occur in eggs than in tissue culture systems. Some recent work with the fluorescent antibody technique (Deibel and Hotchin, 1959) has shown that, with a similar inoculum of influenza virus, calf kidney cells showed a much lower proportion of fluorescent cells than chick embryo cells. Davenport (1952) has suggested that the varying capacity of adapted and unadapted lines of virus to produce cell injury might be explained by a difference in the affinity of these lines of virus for an essential cellular constituent; the rate of synthesis of cell material and withdrawal of this by the virus may determine the fate of the cell and in turn the host. It may be recalled that although influenza virus will multiply readily in many primary cultures, it will undergo only an incomplete or abortive cycle of multiplication in continuous cell lines, e.g. in HeLa cells. This suggests that, to attain infectivity, the virus depends on some intrinsic property of the cell which has been changed during repeated culture. This factor is probably related to the nucleus.

Results with the fluorescent antibody technique, employing large and small inocula of influenza virus.

The fluorescent antibody technique has revealed that part of the multiplication of influenza virus takes place in the nucleus (Watson and Coons, 1954; Liu, 1955). In the present investigation, which was carried out in bovine embryo kidney cells infected with an Asian strain of influenza virus, it was possible, by means of specific antisera, to demonstrate the initial

development of the S antigen in the nucleus followed closely by the appearance of V antigen in the cytoplasm. When the cells were examined at intervals following infection with a small dose of virus, fluorescence due to S antigen was first detectable in the nucleus 6 hours after infection; in later preparations the fluorescence gained in intensity, becoming very bright and filling the nucleus. V antigen was usually not detectable until 8 hours, by which time S antigen could also be observed as weak perinuclear staining in the cytoplasm. These changes were accompanied by an increase in infectious and haemagglutinating virus. The demonstration, by means of fluorescent antibody, that the S antigen was demonstrable before the V antigen is in agreement with the results obtained by Hoyle (1950) from experiments in the chorioallantoic membrane of chick embryos; S antigen was measurable by complement fixation at 2 hours and about one hour earlier than any detectable increase in haemagglutinin. The later detection of S antigen in the present experiments may be explained by differences in the systems employed and in the sensitivity of the techniques.

The results obtained in the present investigation appear similar to those obtained by Breitenfeld and Schäfer (1957) in a study of the development of fowl plague virus in chick embryo tissue culture by means of fluorescein-labelled antisera. The RNA-containing g- antigen was found to develop in a pattern comparable to that of the S antigen of influenza A virus, being first detectable in the nucleus and later appearing in the cytoplasm. The haemagglutinins of fowl plague virus resembled

the V antigen of influenza virus in that they were detected after the g-antigen but they occurred in the entire cell, thus differing from the influenza V antigen, which was only observed in the cytoplasm apart from well defined nuclear foci.

On the basis of their findings, Breitenfeld and Schäfer (1957) were able to put forward the following concept to explain the intracellular multiplication of fowl plague virus: "the S antigen which contains the nucleic acid and therefore represents the carrier of genetic information becomes associated with the host cell nucleus where it is newly synthesised as the first specific virus structure. Later it appears to induce the production of haemagglutinin in the cytoplasm and especially in a locus near the nucleus which has not yet been identified. The two virus components then diffuse towards the periphery of the cell and here combine to form new elementary particles."

More recently, Mueller et al. (1960) studied the incorporation of leucine-C^{14} into S antigen in homogenates of chick embryo cells infected with fowl plague virus. The S antigen labelling activity was not demonstrable until 2 hours after infection, which agreed with previous findings from the fluorescent antibody experiments. This suggests that certain processes must take place in preparation for the synthesis of S antigen. On the other hand, a significant portion of the S antigen labelling activity could be recovered in the supernatant after sedimentation of the nuclear fraction. This suggests that the actual synthesis of S antigen might be extranuclear or that the S antigen synthesising system was extracted from the nuclei.

Further experiments are necessary to completely elucidate the problem.

The concept put forward by Breitenfeld and Schäfer to explain the intracellular multiplication of fowl plague virus could be satisfactorily employed to interpret the present results obtained with influenza virus and would be compatible with the theory put forward by Hoyle (1950) to explain the multiplication of influenza virus. A recent electron microscope study of the structure of the myxoviruses (Horne et al., 1960) has revealed considerable morphological similarities between influenza and fowl plague viruses.

In experiments employing large inocula of Asian influenza virus in bovine embryo kidney cells the results obtained differed from those observed when small inocula were used. Egg titrations failed to provide evidence for a significant rise in infectious virus, but a definite increase in haemagglutinins was demonstrable both in culture fluid and in disrupted cells. This resulted in a gradual decrease in ID/HA ratio, and by 24 hours the ratio in the medium was always slightly lower than that achieved in the parallel dilute inocula experiments. In the case of the medium this cannot be considered significant, but in the cells a considerably lower ratio was observed in the series employing a high multiplicity of infection. The possibility of incomplete virus formation is suggested by these results. It seems probable, however, that the lower ratios achieved in the cells were almost entirely due to the presence of intracellular haemagglutinins which had not yet combined with the S component

or with some component in the cell wall to reach full infectivity.

With respect to incomplete virus production following large inocula the results obtained from egg infectivity and haemagglutination titrations were inconclusive. However, in experiments employing the fluorescent antibody technique, a picture was obtained which was easily distinguishable from that observed when dilute inocula were employed. Although appearing in the same sequence, the specific antigens were observed earlier in the high multiplicity experiments, and as was to be expected, many more cells showed fluorescence; S antigen was first detected in the nucleus at 4 hours, whereas the V antigen was detected at 6 hours.

Apart from the alteration in the time factor, the most striking result emerging from the comparative fluorescent experiments was that the intensity of nuclear staining with the whole antigen and with the S antigen was noticeably less in the high multiplicity experiments. The nuclear fluorescence was also distinctive in pattern of distribution in these experiments and consisted of coarse granules which did not fill the nucleus in contrast to the dilute inocula experiments where the nuclei became filled with bright fluorescence. A possible interpretation of these results would seem to be that less S antigen per cell was produced after infection with a large inoculum, indicating the formation of incomplete virus.

It might be argued that the antibody content of the S antiserum was not sufficient to reveal the presumably higher

amount of S antigen present in the cells following infection with a large inoculum of virus. However, Deibel and Hotchin (1959), who applied the fluorescent technique to estimate the number of bovine embryo kidney cells actually infected with a given inoculum, found that the number of fluorescing cells was independent of the complement-fixing titre of the serum within the range 43 to 512. In the present experiments, the complement-fixing titre of the anti-S serum used was 256 and the specific anti-S titre of the whole antiserum was 91. It is therefore reasonable to assume that the strength of the sera was sufficient to reveal the S antigen which might be present even after infection with large inocula.

Evidence has been presented by Lief and Henle (1956 b) from experiments with influenza virus in the chick embryo, that a reduced amount of S antigen is present in incomplete virus. This did not seem to be due to depressed production of S antigen in the tissues, because similar amounts of S antigen were found to be produced under conditions favouring incomplete virus production as were produced under conditions favouring complete virus production; it appeared rather to be the result of a failure of incorporation of the antigen into the virus particle. Although no definite indication of incomplete virus formation was obtained in our experiments, the considerable reduction in specific staining with anti-S serum, which was observed with a high multiplicity of infection would suggest that less S antigen per cell was being produced. It is difficult to assess the

importance of the apparent discrepancies between our findings and those described by Lief and Henle (1956), particularly when the fundamental differences in the systems employed are taken into consideration.

Bovine embryo kidney cells, like the chick embryo, will support the complete replication of influenza virus under normal conditions. HeLa cells, on the other hand, will only support an incomplete cycle of multiplication. However, by means of the fluorescent antibody technique, it was possible to detect both S and V antigens in influenza-infected HeLa cells; the distribution of S antigen in the nucleus was, in fact, very similar to that observed in bovine embryo kidney cells, where a high multiplicity of infection was employed. Again these results appear to be inconsistent with the studies of Lief and Henle (1956) which indicated that there was no lack of production of S antigen in the tissues when incomplete virus was produced. It is possible that when these authors disrupted the cells, S antigen, measurable by their complement-fixation tests, was released from some intracellular "binding".

In contrast to the results obtained in bovine embryo kidney cells it was observed that the vast majority of infected HeLa cells failed to exhibit the presence of any S antigen in the cytoplasm detectable by the fluorescent techniques employed. This finding is consistent with that of Franklin and Breitenfeld (1959) obtained from an investigation of the abortive infection of Earle's L cells by fowl plague virus. These workers suggested that the formation of incomplete virus in this system

might be related to a barrier existing in the nuclear membrane which cannot be passed by complete S antigen. A somewhat similar explanation was given by Fraser et al. (1959) to account for the inability of non-neurotropic strains of influenza virus to multiply to completion in the mouse brain. On the other hand, it must be noted that some HeLa cells observed in the present experiments demonstrated scattered S antigen in the cytoplasm. Further investigations employing chemical studies must be carried out to support or exclude the possibility that this S antigen differs from that produced under "normal" conditions.

A hypothesis to explain incomplete virus production.

It appears from the results of other workers and from the present study that two types of incomplete virus exist. The type produced during abortive infections, for example in HeLa cells, seems to occur as a result of some fault in the release of virus from the cell. The other type, exemplified by the von Magnus phenomenon, in which incomplete virus is produced in a host which normally supports a complete cycle of multiplication, appears to be formed by some interference mechanism. Interference has long been considered the prime mechanism responsible for the occurrence of an incomplete cycle of reproduction induced by large inocula. Recently, Paucker and Henle (1958) reported that virus will not interfere below a certain level of S antigen content. Considering this finding and the present observation that less S antigen per cell is apparently produced when large inocula are employed, a hypothesis for the formation of incomplete virus is put forward:

When cells are exposed to large quantities of influenza

virus interference occurs and the progeny consist of a certain proportion of incomplete particles deficient in S antigen; after continued undiluted passage the S antigen content decreases further until the virus is incapable of inducing interference. At this stage complete virus is again produced.

This hypothesis appears to provide an adequate explanation of the events occurring during the von Magnus phenomenon and it seems permissible to suggest that the difficulty in detecting such a phenomenon in tissue cultures was due to lower titres obtained in the system used in the present study. With less virus it was impossible to induce such striking interference in the cells as could be achieved in the cells lining the allantoic cavity of the chick embryo.

The production of incomplete virus by influenza virus is thus closely associated with the nucleus and is highly sensitive to experimental conditions and to the system employed. It is therefore not unexpected that Newcastle disease virus, which is known to differ considerably from influenza virus, has so far not been found to undergo an incomplete cycle of multiplication.

SUMMARY

SUMMARY

The relationship between host cells and members of the Myxovirus group has been reviewed with particular reference to the growth of these viruses in tissue culture. Virus multiplication, incomplete virus production and the phenomenon of interference have also been considered. It became apparent from the literature that certain members of the group, for example, Newcastle disease virus (NDV) and influenza, differ significantly from each other in various growth characteristics. It was considered advantageous to study further the differences between these two viruses and their growth characteristics were therefore investigated in various tissue culture systems.

The experiments were planned to study:-

(a) the cytopathic effect of these viruses on their host cells and the relationship between this effect and the production of new virus,

(b) cell survival following infection,

(c) the possibility of incomplete virus formation.

During the course of the study it became possible to investigate in addition the intracellular development of influenza virus.

(a) Cytopathic effect

The cytopathic effect of NDV in HeLa cells was examined by ordinary microscopy and by time-lapse cinematography using interference microscopy. The changes associated with infection could be divided into three phases, namely the appearance in the cell monolayer of circular foci of infection

termed "microplaques", the ensuing formation of syncytial masses and finally complete cellular degeneration.

The cytopathic effect of influenza virus was studied in monkey kidney and bovine embryo kidney cells. The changes differed from those observed with NDV and included vacuolation and granulation of the cytoplasm and the appearance of intra-nuclear vacuoles. Nuclear changes were also observed with the acridine orange technique; the extent and pattern of these changes depended on the strain of virus used.

With both viruses, when small inocula were used, the cytopathic effects were accompanied by an increase in infective and haemagglutinating virus.

(b) Cell survival following infection

Cells subjected to a large dose of NDV survived better than cells similarly treated with influenza virus. It is suggested that this may be due to a difference in toxicity of the viruses. In further experiments, incomplete influenza virus, which is known to be less toxic, produced a less extensive cytopathic effect.

Treatment of the cells with immune serum following infection with NDV reduced cytopathic changes.

(c) Incomplete virus formation

Although infection of HeLa cells with large inocula of NDV did not result in the production of detectable infective virus no evidence for the formation of incomplete virus was obtained.

With influenza virus, on the other hand, evidence was obtained for the production of incomplete virus following undiluted serial passage in monkey kidney cells. The phenomenon was, however, never as striking as that observed in eggs. It is suggested that this may be due to the lower titres obtained in tissue cultures.

The intracellular development of influenza virus

By means of the fluorescent antibody technique using specific antisera, the S and V antigens of influenza virus could be detected in bovine embryo kidney cells and in HeLa cells. The S antigen was first detected in the nucleus whereas the V antigen appeared slightly later in the cytoplasm. It seems feasible that in the case of influenza-infected bovine embryo kidney cells the S and V antigens combine at the surface of the cell prior to their release as complete virus. With HeLa cells S and V antigens are produced in the cells but only incomplete virus is released.

When large inocula were employed, nuclear fluorescence with S antiserum was never as intense as that observed in cells infected with a small dose of virus. This indicates that less S antigen per cell was produced when large inocula were employed and suggests the formation of incomplete virus.

On the basis of these findings a hypothesis has been put forward to explain the formation of incomplete influenza virus. As this phenomenon is so intimately linked to the

relationship between virus and host cell it is conceivable that NDV, which has been found to differ from influenza virus in many growth characteristics, should not be able to induce the production of incomplete virus.

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